

Biocatalysis in the Pharmaceutical and Biotechnology Industries

Series

Biocatalysis in the Pharmaceutical and Biotechnology Industries

Edited by
Ramesh N. Patel



CRC Press

Taylor & Francis Group

Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an informa business

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

© 2007 by Taylor & Francis Group, LLC
CRC Press is an imprint of Taylor & Francis Group, an Informa business

No claim to original U.S. Government works
Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number-10: 0-8493-3732-1 (Hardcover)
International Standard Book Number-13: 978-0-8493-3732-1 (Hardcover)

This book contains information obtained from authentic and highly regarded sources. Reprinted material is quoted with permission, and sources are indicated. A wide variety of references are listed. Reasonable efforts have been made to publish reliable data and information, but the author and the publisher cannot assume responsibility for the validity of all materials or for the consequences of their use.

No part of this book may be reprinted, reproduced, transmitted, or utilized in any form by any electronic, mechanical, or other means, now known or hereafter invented, including photocopying, microfilming, and recording, or in any information storage or retrieval system, without written permission from the publishers.

For permission to photocopy or use material electronically from this work, please access www.copyright.com (<http://www.copyright.com/>) or contact the Copyright Clearance Center, Inc. (CCC) 222 Rosewood Drive, Danvers, MA 01923, 978-750-8400. CCC is a not-for-profit organization that provides licenses and registration for a variety of users. For organizations that have been granted a photocopy license by the CCC, a separate system of payment has been arranged.

Trademark Notice: Product or corporate names may be trademarks or registered trademarks, and are used only for identification and explanation without intent to infringe.

Library of Congress Cataloging-in-Publication Data

Biocatalysis in the pharmaceutical and biotechnology industries / edited by Ramesh N. Patel.
p. ; cm.

Includes bibliographical references and index.

ISBN-13: 978-0-8493-3732-1 (hardcover : alk. paper)

ISBN-10: 0-8493-3732-1 (hardcover : alk. paper)

1. Enzymes--Biotechnology. 2. Biotechnology--Industrial applications. I. Patel, Ramesh N., 1942-
[DNLN: 1. Catalysis. 2. Enzymes. 3. Biotechnology--methods. 4. Pharmaceutical

Preparations--chemical synthesis. QU 135 B6145 2006]

TP248.65.E59B56 2006
660.6'34--dc22

2006008932

Visit the Taylor & Francis Web site at
<http://www.taylorandfrancis.com>

and the CRC Press Web site at
<http://www.crcpress.com>

Preface

There has been an increasing awareness of the enormous potential of microorganisms and enzymes for the transformation of synthetic chemicals in a highly chemo-, regio-, and enantioselective manner. Chiral intermediates are in high demand from pharmaceutical, agricultural, and other biotechnological industries for the preparation of bulk drug substances or fine chemicals. Bulk drug compounds or other fine chemicals can be produced by chemical or chemo-enzymatic synthesis. The advantages of biocatalysis over chemical synthesis are that enzyme-catalyzed reactions are often highly enantio- and regioselective. They can be carried out at ambient temperature and atmospheric pressure, thus avoiding the use of more extreme conditions which could cause problems with isomerization, racemization, epimerization, and rearrangement. Microbial cells and enzymes derived from microbial cells can be immobilized and reused for many cycles. Biocatalysis includes fermentation, biotransformation by whole cells or enzyme-catalyzed transformations, cloning and expression of enzymes, and directed evolution of enzymes to improve selectivity, substrate specificity, and stability. Various chapters in this book are contributed by internationally well-known scientists having many years of experience in different aspects of biocatalysis and biocatalytic applications in production of fine chemicals and chiral pharmaceutical intermediates.

This book contains 34 chapters with over 4000 references and more than 600 tables, equations, drawings, and micrographs. All the information cited in this book provides state-of-the-art knowledge and improves the ability of the reader to use different types of enzymatic reactions in synthesis of fine chemicals and chiral compounds and their application in biotechnological industries. Various chapters discuss the following important aspects in biocatalysis and its applications in various industries:

- Application of nitrilases and nitrile hydratases in synthesis of fine chemicals that describe cloning and expression of nitrilases and their use in production of chiral and achiral carboxylic acids, regioselective and chemoselective hydrolysis of nitriles, preparation of amides from nitriles, commercialized processes for preparation of nicotinamide, cyanovaleramide, acrylamide, and nitrile-containing polymers
- Biocatalytic deracemization processes that include dynamic kinetic resolution, stereoinversion processes, and enantioconvergent processes to prepare chiral compounds such as amino acids, amines, alcohols, diols, and epoxides in theoretical 100% yields
- Biocatalysis in pharmaceutical industries for synthesis of chiral intermediates and fine chemicals for chemoenzymatic synthesis of drugs such as anticancer, antiviral, antihypertensive, anticholesterol, anti-infective, anti-inflammatory, antianxiety, and antipsychotic drugs
- Methods for directed evolution of lipases and esterases, assay development and screening of mutants for selection in esterification, transesterification, acylation and acyl hydrolytic reactions, and use of improved enzymes in organic synthesis
- Oxidative biocatalysis catalyzed by flavin-containing flavoprotein oxidases such as alcohol oxidases, amine oxidases, and sulfhydryl oxidases together with flavoprotein monooxygenases such as aromatic, heteroatom, and multicomponent monooxygenase in enzymatic oxygenation reactions

- Biotransformation (hydroxylation, dealkylation, N-oxide formation, and *O*-demethylation reactions) of natural and synthetic compounds for the generation of molecular diversity and drug metabolites
- Enzymatic acylation of alcohols and amines in preparation of pharmaceuticals such as anticonvulsant agents, anticancer agents, immunosuppressive compounds, analgesic drugs, antidepressants, anticholesterol drugs, antibiotics, β -adrenergic blockers, calcium channel blockers, serotonin uptake inhibitors, antifungal agents, anti-Alzheimer's agents, antiulcer agents, α -adrenoceptor agonists, and other drug substances
- Enzyme-metal complex-catalyzed asymmetric biotransformations and dynamic resolution processes to prepare chiral alcohols, amines, and acetates
- Applications of aromatic hydrocarbon dioxygenases such as toluene dioxygenase, naphthalene dioxygenase, chlorobenzene dioxygenase in synthesis of fine chemicals, and pharmaceuticals with continuous cofactor regeneration during biotransformations
- Investigation on baker's yeast reduction processes by genomic approach and preparation of chiral alcohols and synthesis of anticancer, anticholesterol, and antihypertensive drugs
- Techniques and applications of immobilization of enzymes as cross-linked enzyme aggregates (CLEA) in synthesis of fine chemicals
- Application of C-C bond forming enzymes such as aldolases and transketolases in synthesis of fine chemicals and pharmaceuticals
- Biocatalytic synthesis of nucleoside analogs by modification of base, sugar and oxidation reactions, and applications of nucleoside analogs as antiviral agents
- Biocatalytic reduction of carboxylic acids by carbonyl reductases, mechanism of carbonyl reductases, and cloning, and expression of enzyme with application in synthesis of fine chemicals
- Application of dehalogenases in biocatalysis and biodegradation with emphasis on haloalkane dehalogenases, haloacid dehalogenases, and halohydrin dehalogenases and their application in preparation of chiral compounds
- Enzymatic synthesis of sugar esters and oligosaccharides from renewable resources that includes regioselective synthesis of fatty acid sugar ester using lipases and proteases, and synthesis of oligosaccharides by transglycosidases and transglucosidases
- Efficient methodology and instrumentation for engineering custom enzymes using directed evolution and solid-phase screening to maximize high throughput and selection of evolved enzymes and application of highly active enzymes in synthesis of intermediates for pharmaceuticals
- Biocatalytic enantioselective and diastereoselective deaminations for chemoenzymatic synthesis of antiviral agents using adenosine deaminase or adenylyl deaminase
- Enzymatic resolution of lactones by lactonases and synthesis of chiral alcohols by carbonyl reductases and effective cofactor regenerating systems for synthesis of pharmaceutical intermediates
- Enzymatic acyloin condensations by decarboxylases and rational design of arylmalonate decarboxylase for synthesis of fine chemicals and chiral intermediates
- Enantioselective biocatalysis for synthesis of pheromones and juvenile hormones
- Stereoselective and regioselective modifications of polyhydroxylated steroids by dehydrogenases, lipases, and proteases to prepare novel steroidal compounds
- Industrial enzymatic processes for C-C, C-N, C-O bond formations by lyases such as phenylalanine ammonia lyase, fumarase, malease, hydratases, and dehydratases
- State of the art and application in enantioselective synthesis of chiral cyanohydrins by hydroxynitrile lyases

- Chiral switches strategies, opportunities, and experiences, and biocatalysis in preparation of ibuprofen, ketoprofen, esomeprazole, methylphenidate, doxazosin, levofloxacin, and other chiral molecules
- Cutting-edge methodology for gene shuffling, family of genes shuffling, directed evolution, and high-throughput screening of mutants to increase the selectivity, activity, and stability of enzymes
- Biocatalytic preparations of chiral amines by kinetic resolution, dynamic kinetic resolution; deracemization and stereoinversion processes using transaminases, amine oxidases, and lipases

Biocatalysis in the pharmaceutical and biotechnological applications is an indispensable resource for organic chemists, biochemists, microbiologists, biochemical engineers, biotechnologists, medicinal chemists, pharmacologists, and upper-level undergraduate and graduate students in these disciplines.

It is my pleasure to acknowledge sincere appreciation to all the authors for their contribution to this book. I would like to acknowledge continual support from David Fausel (production coordinator) and Anita Lekhwani (acquisition editor) and Richard Tressider (project editor) of the Taylor & Francis Group and Vinithan Sethumadhavan (project manager) of SPi. My interest in biocatalysis was developed and stimulated by David Gibson, Derek Hoare, Nicholas Ornston, Allen Laskin, Ching Hou, Laszlo Szarka, Christopher Cimarusti, John Scott, Richard Mueller, and many of my colleagues at the University of Texas, Yale University, Exxon Research and Engineering Company, and Bristol-Myers Squibb. I acknowledge their support and encouragement over the last 35 years. Finally, I would like to express my sincere thanks to my wife, Lekha, and my daughter, Sapana, for their support and encouragement while I worked on this book.

Editor

Ramesh N. Patel is a senior research fellow in charge of the Enzyme Technology Department in Process Research and Development at the Bristol-Myers Squibb Pharmaceutical Research Institute, New Brunswick, New Jersey. He was a National Institute of Health and American Chemical Society Postdoctoral Fellow (1971–1975) in the biology department at Yale University, New Haven, Connecticut, and a research scientist (1975–1987) in the biotechnology department at the Corporate Research Center, Exxon Research and Engineering Company, Clinton, New Jersey. Holder of over 70 U.S. and European patents, and author and coauthor of over 150 journal articles and 80 national and international conference presentations, he is a member of the American Association for the Advancement of Science, American Chemical Society, American Society for Microbiologists, Society for Industrial Microbiologists, and the American Oil Chemist's Society. He was awarded the 2004 Biotechnology Lifetime Achievement Award from the Biotechnology Division of the American Oil Chemist's Society. Dr. Patel received his bachelor's degree from Bombay University, Bombay, India; his master's degree from Maharaja Sayajirao University, Baroda, India; and his Ph.D. degree (1971) from the University of Texas, Austin.

Contributors

Yangsoo Ahn

Department of Chemistry
Pohang University of Science and
Technology
Pohang, Korea

M. Alcalde

Department of Biocatalysis
Institute of Catalysis and Petrochemistry
CSIC
Madrid, Spain

Andrés R. Alcántara

Organic and Pharmaceutical Chemistry
Department
Complutense University
Madrid, Spain

Laura Alessandrini

Department of Preclinical Sciences
LITA Vialba
University of Milan
Milan, Italy

Robert Azerad

Laboratoire de Chimie et Biochimie
Pharmacologiques et Toxicologiques
Université René Descartes-Paris 5
Paris, France

A. Ballesteros

Department of Biocatalysis
Institute of Catalysis and Petrochemistry
CSIC
Madrid, Spain

Uwe T. Bornscheuer

Department of Biotechnology and Enzyme
Catalysis
Institute of Biochemistry
Greifswald University
Greifswald, Germany

Stefan Buchholz

Creavis
Degussa AG
Hanau-Wolfgang, Germany

Reuben Carr

School of Chemistry
University of Manchester
Manchester, United Kingdom

Pierangela Ciuffreda

Department of Preclinical Sciences
LITA Vialba
University of Milan
Milan, Italy

William J. Coleman

KAIROS Scientific Inc.
San Diego, California

Luís A. Condezo

Organic and Pharmaceutical Chemistry
Department
Complutense University
Madrid, Spain

Réne Csuk

Institute of Organic Chemistry
Martin Luther University Halle–Wittenberg
Halle (Saale), Germany

Lacy Daniels

College of Pharmacy
Texas A&H Health Science Center
Kingsville, Texas

Robert DiCosimo

DuPont Central Research and Development
Wilmington, Delaware

Susanne Dreyer

Department of Chemistry
University of Rostock
Rostock, Germany

Franz Effenberger

Institute of Organic Chemistry
University of Stuttgart
Stuttgart, Germany

Kurt Faber

Department of Chemistry
University of Graz
Graz, Austria

Jesús Fernández-Lucas

Organic and Pharmaceutical Chemistry
Department
Complutense University
Madrid, Spain

M. Ferrer

Department of Biocatalysis
Institute of Catalysis and Petrochemistry
CSIC
Madrid, Spain

Wolf-Dieter Fessner

Institute of Organic Chemistry and
Biochemistry
Technical University of Dornstadt
Dornstadt, Germany

Siegfried Förster

Institute of Organic Chemistry
University of Stuttgart
Stuttgart, Germany

Elena Fossati

Institute of Chemistry of Molecular Recognition
Milan, Italy

Marco W. Fraaije

Biochemical Laboratory
Groningen Biomolecular Sciences and
Biotechnology Institute
University of Groningen
Groningen, The Netherlands

H. García-Arellano

Department of Organic and Inorganic
Chemistry
University of Oviedo
CSIC
Madrid, Spain

Carlos A. García-Burgos

Organic and Pharmaceutical Chemistry
Department
Complutense University
Madrid, Spain

I. Ghazi

Department of Organic and Inorganic
Chemistry
University of Oviedo
CSIC
Madrid, Spain

Vicente Gotor

Department of Organic and Inorganic
Chemistry
University of Oviedo
Oviedo, Spain

Vicente Gotor-Fernández

Department of Organic and Inorganic
Chemistry
University of Oviedo
Oviedo, Spain

Harald Gröger

Service Center Biocatalysis
Degussa AG
Hanau-Wolfgang, Germany

Aurelio Hidalgo

Department of Biotechnology and Enzyme
Catalysis
Institute of Biochemistry
Greifswald University
Greifswald, Germany

Michael J. Homann

Schering-Plough Research Institute
Union, New Jersey

Kohsuke Honda

Division of Applied Life Sciences
Graduate School of Agriculture
Kyoto University
Kyoto, Japan

Gjalt W. Huisman

Codexis Inc.
Redwood City, California

Takeru Ishige

Division of Applied Life Sciences
Graduate School of Agriculture
Kyoto University
Kyoto, Japan

Dick B. Janssen

Groningen Biomolecular
Sciences and Biotechnology
Institute
University of Groningen
Groningen, The Netherlands

Stefan Jennewein

Institute of Organic Chemistry and
Biochemistry
Technical University of Dornstadt
Dornstadt, Germany

Michihiko Kataoka

Division of Applied Life Sciences
Graduate School of Agriculture
Kyoto University
Kyoto, Japan

Mahn-Joo Kim

Department of Chemistry
Pohang University of Science and
Technology
Pohang, Korea

Christoph Kobler

Institute of Organic Chemistry
University of Stuttgart
Stuttgart, Germany

Udo Kragl

Department of Chemistry
University of Rostock
Rostock, Germany

Wolfgang Kroutil

Department of Chemistry
University of Graz
Graz, Austria

James J. Lalonde

Codexis Inc.
Redwood City, California

Julia Lembrecht

Department of Chemistry
University of Rostock
Rostock, Germany

Andreas Liese

Institute of Technical Biocatalysis
Technical University of
Hamburg-Harburg
Hamburg, Germany

Mahmoud Mahmoudian

Research and Development
Process Chemicals
Rohm & Haas
Spring House, Pennsylvania

Akinobu Matsuyama

Corporate Development Center
Daicel Chemical Industries Ltd.
Tsukuba, Japan

Kenji Miyamoto

Department of Biosciences and
Informatics
Keio University
Yokohama, Japan

Kenji Mori

RIKEN Research Center for Allergy and
Immunology
Wako, Japan

Hiromichi Ohta

Department of Biosciences and
Informatics
Keio University
Yokohama, Japan

Rebecca E. Paraless

Section of Microbiology
University of California
Davis, California

Jaiwook Park

Department of Chemistry
Pohang University of Science and
Technology
Pohang, Korea

Ramesh N. Patel

Process Research and Development
Bristol-Myers Squibb
New Brunswick, New Jersey

F.J. Plou

Department of Biocatalysis
Institute of Catalysis and Petrochemistry
CSIC
Madrid, Spain

Martina Pohl

Institute of Molecular Enzyme
Technology
Heinrich Heine University of
Düsseldorf
Jülich, Germany

Francisca Rebolledo

Department of Organic and Inorganic
Chemistry
University of Oviedo
Oviedo, Spain

Sol M. Resnick

Biotechnology Core Research &
Development
Dow Chemical Company
San Diego, California

D. Reyes-Duarte

Department of Biocatalysis
Institute of Catalysis and
Petrochemistry
CSIC
Madrid, Spain

Sergio Riva

Institute of Chemistry of Molecular
Recognition
Milan, Italy

Steven J. Robles

KAIROS Scientific Inc.
San Diego, California

John P.N. Rosazza

Center for Biocatalysis and Bioprocessing
University of Iowa
Iowa City, Iowa

Enzo Santaniello

Department of Preclinical Sciences
LITA Vialba
University of Milan
Milan, Italy

Jan Schumacher

Department of Chemistry
University of Rostock
Rostock, Germany

Roger A. Sheldon

Department of Biotechnology
Delft University of Technology
Delft, The Netherlands

Sakayu Shimizu

Division of Applied Life Sciences
Graduate School of Agriculture
Kyoto University
Kyoto, Japan

Yolanda Simeó

Department of Chemistry
University of Graz
Graz, Austria

Ajay Singh

Department of Biology
University of Waterloo
Waterloo, Ontario, Canada

José V. Sinisterra

Organic and Pharmaceutical Chemistry
Department
Complutense University
Madrid, Spain

Jon D. Stewart

Department of Chemistry
University of Florida
Gainesville, Florida

Wen-Chen Suen

Schering-Plough Research
Institute
Union, New Jersey

Nicholas J. Turner
School of Chemistry
University of Manchester
Manchester, United Kingdom

Willem J.H. van Berkel
Laboratory of Biochemistry
Wageningen University
Wageningen, The Netherlands

Padmesh Venkitasubramanian
Center for Biocatalysis and Bioprocessing
University of Iowa
Iowa City, Iowa

Owen Ward
Department of Biology
University of Waterloo
Waterloo, Ontario, Canada

Hiroaki Yamamoto
Lifescience Development Center
CPI Company
Daicel Chemical Industries Ltd.
Tsukuba, Japan

Mary M. Yang
KAIROS Scientific Inc.
San Diego, California

Aleksey Zaks
Schering-Plough Research Institute
Union, New Jersey

Ningyan Zhang
Merck Research Laboratories
West Point, Pennsylvania

Table of Contents

Chapter 1 Nitrilases and Nitrile Hydratases

Robert DiCosimo

Chapter 2 Biocatalytic Deracemization: Dynamic Resolution, Stereoinversion, Enantioconvergent Processes, and Cyclic Deracemization

Yolanda Simeó, Wolfgang Kroutil, and Kurt Faber

Chapter 3 A Decade of Biocatalysis at Glaxo Wellcome

Mahmoud Mahmoudian

Chapter 4 Biocatalysis for Synthesis for Chiral Pharmaceutical Intermediates

Ramesh N. Patel

Chapter 5 Directed Evolution of Lipases and Esterases for Organic Synthesis

Aurelio Hidalgo and Uwe T. Bornscheuer

Chapter 6 Flavin-Containing Oxidative Biocatalysts

Marco W. Fraaije and Willem J.H. van Berkel

Chapter 7 Preparation of Chiral Pharmaceuticals through Enzymatic Acylation of Alcohols and Amines

Vicente Gotor-Fernández, Francisca Rebolledo, and Vicente Gotor

Chapter 8 Dynamic Kinetic Resolution and Asymmetric Transformations by Enzyme–Metal Combinations

Mahn-Joo Kim, Yangsoo Ahn, and Jaiwook Park

Chapter 9 Biotransformation of Natural or Synthetic Compounds for the Generation of Molecular Diversity

Robert Azerad

Chapter 10 Applications of Aromatic Hydrocarbon Dioxygenases

Rebecca E. Parales and Sol M. Resnick

Chapter 11 A Genomic Approach to Investigating Baker's Yeast Reductions

Jon D. Stewart

Chapter 12 Immobilization of Enzymes as Cross-Linked Enzyme Aggregates: A Simple Method for Improving Performance

Roger A. Sheldon

Chapter 13 Biotechnological Applications of Aldolases

Wolf-Dieter Fessner and Stefan Jennewein

Chapter 14 Enzymatic Synthesis of Modified Nucleosides

Luis A. Condezo, Jesús Fernández-Lucas, Carlos A. García-Burgos, Andrés R. Alcántara, and José V. Sinisterra

Chapter 15 Biocatalytic Reduction of Carboxylic Acids: Mechanism and Applications

Padmesh Venkitasubramanian, Lacy Daniels, and John P.N. Rosazza

Chapter 16 Dehalogenases in Biodegradation and Biocatalysis

Dick B. Janssen

Chapter 17 Enzymatic Synthesis of Sugar Esters and Oligosaccharides from Renewable Resources

A. Ballesteros, F.J. Plou, M. Alcalde, M. Ferrer, H. García-Arellano, D. Reyes-Duarte, and I. Ghazi

Chapter 18 Efficient Methods and Instrumentation for Engineering Custom Enzymes

Steven J. Robles, William J. Coleman, and Mary M. Yang

Chapter 19 Deaminating Enzymes of the Purine Cycle as Biocatalysts for Chemoenzymatic Synthesis and Transformation of Antiviral Agents Structurally Related to Purine Nucleosides

Enzo Santaniello, Pierangela Ciuffreda, and Laura Alessandrini

Chapter 20 Microbial and Enzymatic Processes for the Production of Chiral Compounds

Kohsuke Honda, Takeru Ishige, Michihiko Kataoka, and Sakayu Shimizu

Chapter 21 Discovery of Arylmalonate Decarboxylase and Conversion of the Function by Rational Design

Kenji Miyamoto and Hiromichi Ohta

Chapter 22 Chemoenzymatic Preparation of Enantiopure Building Blocks of Synthetic Utility

Kenji Mori

Chapter 23 Stereoselective Modifications of Polyhydroxylated Steroids

Elena Fossati and Sergio Riva

Chapter 24 Recent Developments in Enzymatic Acyloin Condensations

Owen Ward and Ajay Singh

Chapter 25 Synthesis of Chiral Alcohols with Carbonyl Reductase
Library and Robust NAD(P)H Regenerating System

Hiroaki Yamamoto and Akinobu Matsuyama

Chapter 26 Comparative Analysis of Chemical and Biocatalytic
Syntheses of Drug Intermediates

Michael J. Homann, Wen-Chen Suen, Ningyan Zhang, and Aleksey Zaks

Chapter 27 Industrial Processes Using Lyases for C–C, C–N,
and C–O Bond Formation

Martina Pohl and Andreas Liese

Chapter 28 State of the Art and Applications in Stereoselective
Synthesis of Chiral Cyanohydrins

Franz Effenberger, Siegfried Förster, and Christoph Kobler

Chapter 29 Chiral Switches: Problems, Strategies,
Opportunities, and Experiences

René Csuk

Chapter 30 Enzyme Evolution for Chemical Process Applications

Gjalt W. Huisman and James J. Lalonde

Chapter 31 Biocatalytic Routes to Nonracemic Chiral Amines

Nicholas J. Turner and Reuben Carr

Chapter 32 Enantioselective Biocatalytic Reduction of Ketones
for the Synthesis of Optically Active Alcohols

Stefan Buchholz and Harald Gröger

Chapter 33 Enzyme Catalysis in Nonaqueous Media: Past, Present, and Future

Susanne Dreyer, Julia Lembrecht, Jan Schumacher, and Udo Kragl

Chapter 34 Biocatalytic Concepts for the Synthesis of Optically Active Amines

Stefan Buchholz and Harald Gröger

1 Nitrilases and Nitrile Hydratases

Robert DiCosimo

CONTENTS

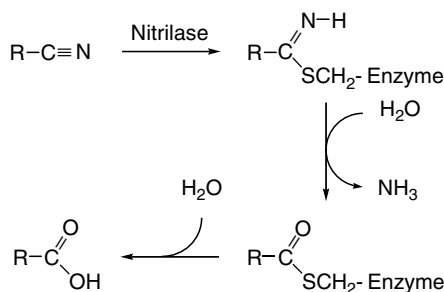
1.1	Introduction	1
1.2	Nitrilases	1
1.2.1	Heterologous Nitrilase Expression	2
1.2.2	Production of Chiral Carboxylic Acids	3
1.2.3	Regioselective and Chemoselective Nitrile Hydrolysis	5
1.2.4	Achiral Carboxylic Acids	8
1.2.5	Amides via Nitrilase Catalysis	8
1.3	NHase and NHase/Amidase	9
1.3.1	Crystal Structures	10
1.3.2	Heterologous Protein Expression	10
1.3.3	Enantioselective Nitrile Hydrolysis	10
1.3.4	NHase/Amidase Catalysts for Production of Achiral Carboxylic Acids	15
1.3.5	Commercialized Processes Using NHase Catalysts	16
1.3.5.1	Nicotinamide (Niacinamide)	16
1.3.5.2	5-Cyanovaleramide	16
1.3.5.3	Acrylamide	17
1.3.6	Modification of Nitrile-Containing Polymers	19
1.3.7	Nitrilase or NHase?	19
1.4	Conclusions	20
	References	20

1.1 INTRODUCTION

Nitrilase and nitrile hydratase (NHase) are two classes of enzymes that are finding increasing use as catalysts for the conversion of nitriles to carboxylic acids and amides, respectively; in addition, NHases are often used in combination with amidases to produce carboxylic acids. These reactions can be enantioselective, chemoselective, and/or regioselective, and there are often no equivalent chemical catalysts that can produce the desired product with the same selectivity afforded by the enzyme-catalyzed reactions. A large number of publications, patent applications, and patents describe the preparation and use of these enzyme catalysts, and numerous reviews of nitrilase- and NHase-catalyzed reactions have been published. The most recent work in this area has been summarized here, with a focus on the application of these enzymes as catalysts in synthesis, as well as in process development or in commercial processes.

1.2 NITRILASES

Nitrilases have been the subject of a number of recent reviews [1–7]. All known nitrilases share a highly conserved region of amino acid sequence which includes a cysteine that is responsible for the catalytic activity of the enzyme [1,8]. The mechanism that has been proposed for conversion



SCHEME 1.1 Proposed mechanism for hydrolysis of nitrile to carboxylic acid by nitrilase. (Redrawn from Kobayashi, M., Goda, M., and Shimizu, S., *Biochem. Biophys. Res. Commun.*, 253, 662, 1998.)

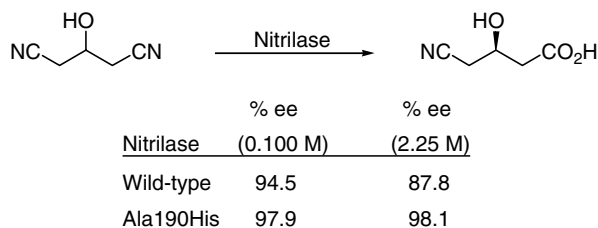
of a nitrile to a carboxylic acid by nitrilase is depicted in Scheme 1.1, where, after binding in the enzyme active site, the nitrile reacts with a cysteine sulfhydryl residue to produce an intermediate thioimidate; subsequent hydrolysis of this thioimidate produces the ammonium salt of the carboxylic acid [9]. A crystal structure has not yet been reported for nitrilase.

1.2.1 HETEROLOGOUS NITRILASE EXPRESSION

A number of recent patent applications describe the preparation of nitrilase catalysts, where either the nitrilase gene was isolated from a wild-type cell and expressed in a transformant such as *Escherichia coli*, or variants of the wild-type nitrilase gene were created through directed evolution techniques and heterologously expressed. The *nitA* gene encoding an enantioselective nitrilase from *Rhodococcus rhodochrous* NCIMB 11216 was cloned and expressed in *E. coli*, and the resulting transformants screened for activity against a variety of aliphatic and arylaliphatic nitriles [10]. A “PnitA-NitR” system for regulatory gene expression in *Streptomyces* has been developed, based on the expression mechanism of *R. rhodochrous* J1 nitrilase, which is highly induced by ϵ -caprolactam [11]; heterologous protein expression yielded nitrilase levels of as high as 40% of soluble protein. The nitrilase from *Acidovorax facilis* 72W has been cloned and expressed in *E. coli*, where the amount of nitrilase protein produced (active and inactive) was 58% of total soluble protein, and active nitrilase comprised 12% of total soluble protein [12].

The nitrilase gene from the photosynthetic cyanobacterium *Synechocystis* sp. strain PCC6803 has been expressed in *E. coli*, and the purified nitrilase isolated from this recombinant strain was characterized [13]. The observed substrate specificity of the purified nitrilase most closely resembled that of previously described aliphatic nitrilases, and the temperature optima (40 to 45°C) and pH optima (pH 7 to 7.5) were similar to the nitrilases of the mesophilic bacteria *R. rhodochrous* J1 or *Alcaligenes faecalis* JM3. The purified enzyme was active in the presence of a wide range of organic solvents; for example, after incubation of the nitrilase for 10 min in a mixture of 50 mM phosphate buffer and solvent, the rate of hydrolysis of benzonitrile to benzoic acid was not significantly affected by 40% dimethyl sulfoxide or *n*-heptane, 20% methanol, or 10% ethanol or dimethyl formamide. The turnover rates of substrates with poor water solubility, e.g., dodecanoic acid nitrile and naphthalenecarbonitrile, were increased in the presence of both water-soluble and water-immiscible solvents.

Two nitrilase genes, ZmNIT1 and ZmNIT2, have been isolated from maize (*Zea mays*), and heterologously expressed in *E. coli*. [14]. ZmNIT2 and *Arabidopsis* NIT4 have a relatively high homology (69.3%) but ZmNIT2 had no activity toward β -cyanoalanine, the substrate of *Arabidopsis* NIT4, and instead hydrolyzed indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA), where AtNIT4 had no activity for hydrolysis of IAN.

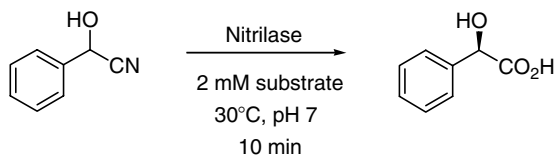


SCHEME 1.2 Desymmetrization of prochiral 3-hydroxyglutaronitrile to (*R*)-3-hydroxy-4-cyanobutyric acid.

1.2.2 PRODUCTION OF CHIRAL CARBOXYLIC ACIDS

A method for the desymmetrization of prochiral 3-hydroxyglutaronitrile using a nitrilase has been demonstrated (Scheme 1.2) [15,16]; esterification of the resulting (*R*)-3-hydroxy-4-cyanobutyric acid produced an intermediate useful for the manufacture of the cholesterol-lowering drug Lipitor (atorvastatin calcium). Nitrilases identified in genomic libraries created by extraction of DNA directly from environmental samples were expressed in *E. coli*, then the resulting library was screened for nitrilases that were highly enantioselective for this reaction [17]. Using one of these (*R*)-specific nitrilases, (*R*)-3-hydroxy-4-cyanobutyric acid was produced using a 100 mM initial nitrile concentration in 98% yield and 94.5% ee. The enantioselectivity of this wild-type nitrilase decreased with increasing nitrile concentration, where only 87.8% ee was obtained using a more industrially relevant substrate concentration of 2.25 M. Mutagenesis of the nitrilase using a technique that combinatorially saturated each amino acid in the protein to each of the other 19 amino acids resulted in an improved variant (Ala190His), which was expressed in *E. coli*; this variant gave an enantiomeric excess of 98.1% and 98.5% at 2.25 M and 3 M substrate concentration, respectively, with a volumetric productivity of 619 g/L/d at 3 M final product concentration. Nitrilases from this library have been used to produce a range of (*R*)-mandelic acid derivatives and analogs, and (*S*)-phenyllactic acid, with high yields and enantioselectivities [16], and a nitrilase from this collection has been expressed in *Pseudomonas fluorescens* [18].

P. putida, *Microbacterium paraoxydans*, and *M. liquefaciens* possess enantioselective nitrilase activity for hydrolysis of (*RS*)-mandelonitrile to (*R*)-(-)-mandelic acid with good specific activity (0.33 to 0.50 U/mg), and high ee (>93%) and *E* values (Scheme 1.3) [19]. *P. putida* was preferred, as it demonstrated a higher reaction rate, lower *K_m*, good yield and ee values, and higher stability compared with the other two microorganisms.



Nitrilase	% Conversion	% ee
<i>Pseudomonas putida</i>	29.2	99.98
<i>Microbacterium paraoxydans</i>	15.3	99.89
<i>Microbacterium liquefaciens</i>	16.6	93.81

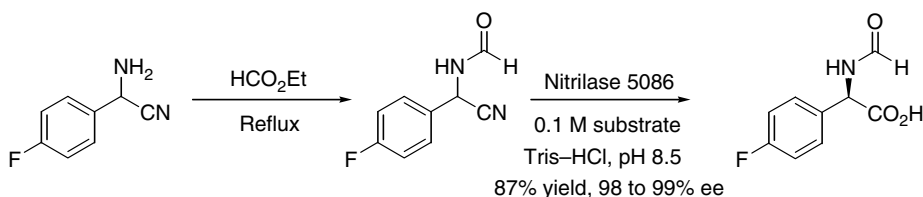
SCHEME 1.3 Conversion of (*RS*)-mandelonitrile to (*R*)-mandelic acid.

Substrate (0.1 M)	Time (h)	Product(s)	Yield (%)
	47		99
	33		82
			6
	115		93
	120		70

SCHEME 1.4 Nitrilase-catalyzed hydrolysis of nitrile precursors of methionine and methionine analogs.

An immobilized *E. coli* transformant expressing the nitrilase from *A. faecalis* (ATCC 8750) [20] was employed in the synthesis of hydroxy analogs of methionine that are useful as nutritional additives in cattle feed (Scheme 1.4) [21,22]. There was no enantioselectivity for hydrolysis of 2-hydroxy-4-methylthiobutanenitrile, 2-acetoxy-4-methylthiobutanenitrile, 2-(1-ethoxyethoxy)-4-methylthiobutanenitrile, or 2-methoxymethoxy-4-methylthiobutanenitrile when compared with the hydrolysis of mandelonitrile, which produced *R*-(-)-mandelic acid in 99% ee at pH 8.5 (presumably by re-equilibration of the cyanohydrin during the course of the conversion). The absence of enantioselectivity for the production of methionine and its hydroxyl analogs was not critical, as the enantiomers are nutritionally equivalent. Modified nitrilases with improved activity for hydrolysis of 2-amino-4-(methylthio)butyronitrile were prepared by making an *A. faecalis* C162N nitrilase mutant and a *Comamonas testosteroni* Q162C nitrilase mutant [23]. 2-Hydroxy-4-methylthiobutyric acid has also been prepared using *R. rhodochrous* B24-1 (FERM P-17515) nitrilase [24].

As an alternative to using an enantioselective nitrilase for the hydrolysis of racemic α -hydroxynitriles to chiral α -hydroxycarboxylic acids, it has been proposed that chiral α -hydroxynitriles (readily prepared by the addition of HCN to aldehydes or ketones using *R*- or *S*-specific oxynitrilases) [25] or their derivatives can be hydrolyzed to the desired chiral α -hydroxycarboxylic acids by nitrilase, or a combination of NHase and amidase [26]. The α -hydroxynitriles are often not stable in aqueous solution, where the cyanohydrin can re-equilibrate with HCN and aldehyde/ketone, resulting in a loss of chirality. Prior conversion of α -hydroxynitriles to the corresponding 2-acetoxynitriles prevented cyanohydrin decomposition or re-equilibration, and the hydrolysis of 2-acetoxybutanenitrile, 2-acetoxyheptanenitrile, 2-acetoxy-2-(2-furyl)acetoneitrile, and 2-acetoxy-2,3,3-trimethylbutanenitrile (ATMB) with several *P. fluorescens* nitrilases was examined. No hydrolysis of the sterically hindered ATMB was observed, and all wild-type strains were found to contain esterases that converted the 2-acetoxynitriles to 2-hydroxynitriles, which then spontaneously decomposed to aldehyde



SCHEME 1.5 Dynamic kinetic resolution of *N*-formyl-4-fluorophenylglycinonitrile to produce (*R*)-*N*-formyl-4-fluorophenylglycine.

and cyanide. Chemoselective hydrolysis to the desired 2-acetoxycarboxylic acids was ultimately achieved using either isolated nitrilase or recombinant *E. coli* strains that heterologously expressed nitrilase activities originating from *P. fluorescens* EBC191, *R. rhodochrous* NCIMB 111216, or *Synechocystis* spp. PCC6803.

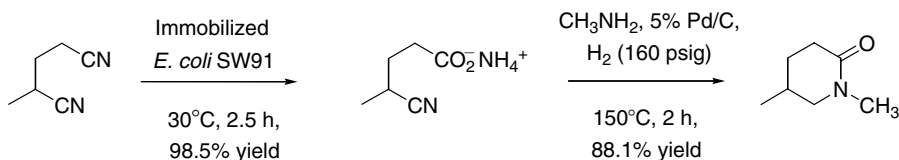
The dynamic kinetic asymmetric hydrolysis of aromatic aminonitriles, phenylglycinonitrile, and 4-fluorophenylglycinonitrile was performed at high pH to produce the corresponding amino acids in high ee [27]. *N*-Acylation of aromatic aminonitriles at pH 8 resulted in spontaneous racemization, allowing enantioselective nitrilase-catalyzed hydrolysis of the (*R*)-enantiomer to produce the corresponding *N*-acylamino acids in up to 99% ee (Scheme 1.5).

New nitrilases with modified substrate/activity profiles were obtained by making a single amino acid change in an *A. faecalis* nitrilase at position 296 such that the amino acid was not tyrosine [28]. Two mutants of *A. faecalis* nitrilase (Y296 → C and Y296 → A) were used for the preparation of substituted chiral carboxylic acids from racemic nitriles, including 2-chloromandelonitrile. The enantioselective preparation of hydroxycarboxylic acids from racemic nitriles, particularly for conversion of 2-chloromandelonitrile to (*R*)-2-chloromandelic acid, was also demonstrated using mutants of *A. faecalis* NitB nitrilase [29].

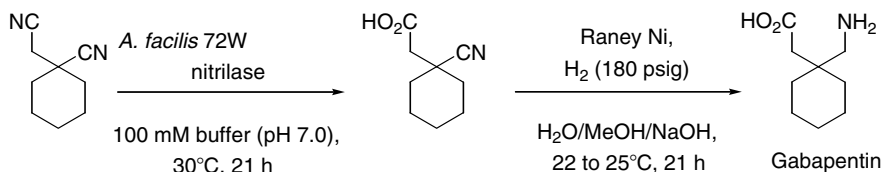
1.2.3 REGIOSELECTIVE AND CHEMOSELECTIVE NITRILE HYDROLYSIS

A. facilis 72W or *E. coli* SW91 (a transformant expressing the 72W nitrilase) cells having a regioselective microbial nitrilase were utilized for conversion of 2-methylglutaronitrile to 4-cyanopentanoic acid (Scheme 1.6), an intermediate in the preparation of 1,5-dimethyl-2-piperidone (an industrial solvent with chemical properties similar to *N*-methylpyrrolidinone) [30–32]. Whole cells were immobilized in alginate beads, and the beads were chemically cross-linked with glutaraldehyde and polyethylenimine before use. An average volumetric productivity of 79 g 4-cyanopentanoic acid/L/h (216 g/L final concentration of the ammonium salt, 98.5% yield at 100% conversion) was achieved over the course of 195 consecutive batch reactions with catalyst recycle using the immobilized *E. coli* SW91 transformant, where the remaining catalyst activity was 67% of initial activity, and catalyst productivity was 3500 g 4-cyanopentanoic acid/g dry cell weight.

1-Cyanocyclohexanecarboxylic acid is an intermediate in the synthesis of gabapentin [33] (1-aminomethyl-1-cyclohexanecarboxylic acid), which has been used for treatment of cerebral



SCHEME 1.6 Regioselective hydrolysis of 2-methylglutaronitrile to 4-cyanopentanoic acid.



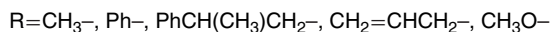
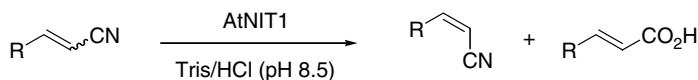
SCHEME 1.7 Regioselective hydrolysis of 1-cyanocyclohexaneacetonitrile to 1-cyanocyclohexaneacetic acid.

diseases. Microbial nitrilase catalysts such as *A. facilis* 72W and *E. coli* SS1001 (a transformant expressing the *A. facilis* 72W nitrilase) have been used for the preparation of 1-cyanocyclohexaneacetic acid by regioselective hydrolysis of 1-cyanocyclohexaneacetonitrile (Scheme 1.7) [34]. Reactions were performed using either unimmobilized cells or calcium alginate-immobilized cells as catalysts, where quantitative conversion of the dinitrile with 100% regioselectivity to the desired product was obtained. For example, immobilized *E. coli* SS1001 cells were used in 35 consecutive batch reactions to prepare 1-cyanocyclohexaneacetic acid with a catalyst productivity of 77 g product/g dry cell weight.

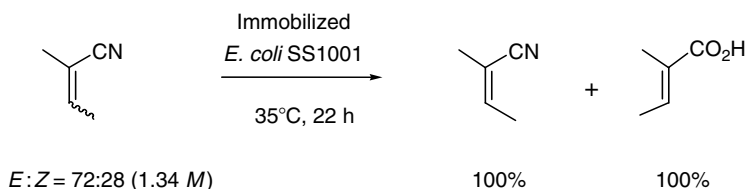
An immobilized *E. coli* transformant expressing an *A. faecalis* nitrilase was highly regioselective for hydrolysis of a series of C₃–C₅ aliphatic dinitriles, producing the corresponding cyano acids, and no diacid, at complete conversion of dinitrile [21]. The regioselective hydrolysis of α,ω -dicyanoalkanes and β -substituted glutaronitriles by *Arabidopsis thaliana* AtNIT1 was examined [35], and selectivity of hydrolysis of dinitriles to ω -cyano-carboxylic acids depended on chain length; at complete conversion of dinitrile the cyano-carboxylic acid was the sole product for chain length of up to six carbon atoms. Glutaronitriles were hydrolyzed to the corresponding cyanobutanoic acids with 100% selectivity. Aromatic poly-nitriles such as orthophthalonitrile, isophthalonitrile, and terephthalonitrile were converted to the corresponding cyanocarboxylic acids using a microbial catalyst prepared by the recombinant expression of the *Rhodococcus* sp. ATCC 89484 nitrilase in *E. coli* [36,37].

R. rhodochrous LL100-21 expressed different nitrile-hydrolyzing enzymes, depending on the mononitrile used as nitrogen source during growth [38]. Cell suspensions grown on propionitrile or benzonitrile converted only the aliphatic group of 2-(cyanomethyl)benzonitrile, producing 2-(cyanophenyl)acetic acid as the sole reaction product, purportedly by the action of a regioselective nitrilase. In contrast, 3-(cyanomethyl)benzonitrile and 4-(cyanomethyl)benzonitrile were converted to 3- and 4-(cyanomethyl)benzoic acid, respectively, as the major hydrolysis product, although small amounts of the corresponding diacids were also observed. The regioselectivity of the nitrilase for 3- and 4-(cyanomethyl)benzonitriles differs markedly from the 2-cyano derivative, where the aliphatic side chain of the 2-cyano derivative may have prevented hydrolysis of the aromatic cyano group due to steric hindrance. The aliphatic cyano group of 3- and 4-(cyanomethyl)benzoic acid was not hydrolyzed, suggesting that the nitrilase enzyme could only hydrolyze this cyano group when there is a cyano group (or other substituent) in the *ortho* position. The same cells, when grown on acetonitrile, converted 2-(cyanomethyl)benzonitrile to the amides 2-(cyanophenyl)acetamide and 2-(cyanomethyl)benzamide in low yields; in this case, hydrolysis was the result of NHase activity.

AtNIT1 hydrolyzed the (*E*)-isomers of stereoisomeric α,β -unsaturated nitriles exclusively to the corresponding (*E*)-carboxylic acids with high specificity, thereby also making possible the preparation of isomerically pure (*Z*)-nitriles (Scheme 1.8) [39]. (*E*)-Selectivity was not obtained when the location of the double bond was changed from α,β -unsaturation to



SCHEME 1.8 Regioselective hydrolysis of stereoisomeric α,β -unsaturated nitriles to the corresponding (*E*)-carboxylic acids.



SCHEME 1.9 Regioselective hydrolysis of (*E,Z*)-2-methyl-2-butenenitrile to (*E*)-2-methyl-2-butenic acid.

β,γ -unsaturation (e.g., for hydrolysis of (*E,Z*)-3-heptenenitrile). *A. facilis* 72W nitrilase (expressed in *E. coli* SS1001) exhibited a similar stereoisomeric preference, where for the regioselective hydrolysis of (*E,Z*)-2-methyl-2-butenenitrile, only (*E*)-2-methyl-2-butenic acid was produced at complete conversion of the (*E*)-nitrile (Scheme 1.9) [40]. Solvent extraction of the unreacted (*Z*)-nitrile from the aqueous product mixture containing the (*E*)-acid ammonium salt, followed by acidification of the aqueous phase and solvent extraction of the resulting (*E*)-acid, allowed for the simple separation and isolation of both reaction products in high yield and purity.

Nitrilases can also be highly chemoselective; *A. facilis* 72W nitrilase converted aliphatic or aromatic cyanocarboxylic acid esters to the corresponding dicarboxylic acid monoesters at 100% conversion with little or no hydrolysis of the ester functionality (Table 1.1) [41]. The microbial cells were first heat-treated at 50°C for 1 h to inactivate a co-expressed NHase

TABLE 1.1
Chemoselective Hydrolysis of Cyanocarboxylic Acid Esters Using *A. facilis* 72W Microbial Nitrilase

Cyanocarboxylic Acid Ester	Concentration (M)	Dicarboxylic Acid Monoester	Yield (%)
Cyanoacetic acid	0.40	Propanedioic acid	100
Ethyl ester		Monoethyl ester	
Cyanoacetic acid	0.40	Propanedioic acid	100
Propyl ester		Monopropyl ester	
3-Cyanopropanoic acid	2.00	Butanedioic acid	100
Acid methyl ester		Monomethyl ester	
5-Cyanopentanoic acid	1.00	Hexanedioic acid	97
Acid methyl ester		Monomethyl ester	
4-Cyanobenzoic acid	0.052	1,4-Benzenedicarboxylic acid	94
Methyl ester		Acid monomethyl ester	

activity (with no loss of nitrilase activity), then 1.2 g dry cell weight/L of unimmobilized cells were used to convert 3-cyanopropanoic acid methyl ester (226 g/L) to butanedioic acid monomethyl ester in 100% yield in 2 h at 25°C.

A general survey of a variety of arylacetonitrilases has been performed using a range of aromatic and arylaliphatic nitrile substrates [42]. Each biocatalyst exhibited a unique substrate-conversion profile, except that α -substituted arylaliphatic nitriles were generally not readily converted to the corresponding carboxylic acids. Chemoselective hydrolysis of the nitrile functionality of substrates having both nitrile and ester groups was demonstrated, as was the regioselective hydrolysis of single nitrile groups in dinitrile substrates. The biocatalysts also mediated the synthesis of a range of α -hydroxycarboxylic acids from aldehydes in the presence of cyanide.

1.2.4 ACHIRAL CARBOXYLIC ACIDS

Several microbial cell catalysts for production of ammonium acrylate have been reported. A Rhodococcal isolate having only a combination of NHase and amidase was compared to *R. ruber* NCIMB 40757, which has only an aliphatic nitrilase [43]. The microbial nitrilase catalyst was preferred, where the nitrilase was much more stable in the presence of acrylonitrile and ammonium acrylate; a fed-batch reaction produced ~500 g product/L. Polyacrylamide-immobilized *Acinetobacter* sp. AK226 was used to produce 300 g/L of ammonium acrylate from acrylonitrile with a catalyst productivity of 4000 g product/g dry cell weight; the reaction was run in the absence of buffer, thereby producing a very pure aqueous reaction product [44]. *R. rhodochrous* NCIMB 40757 and NCIMB 40833 each had a K_m for acrylonitrile below 500 μ M and a K_i for ammonium acrylate above 100,000 μ M; immobilized cells were used in either continuous or fed-batch reactions to produce 400 g/L product with less than 0.2% acrylonitrile remaining [45]. Screening isolates from environmental samples for tolerance to acrylonitrile identified *A. denitrificans* C-32 as a nitrilase-containing strain with high activity for hydrolysis of acrylonitrile [46].

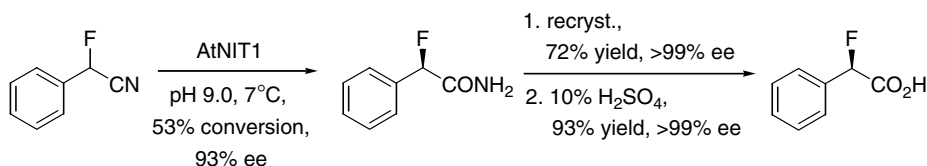
The nitrilase activity of *A. facilis* 72W whole cells, or *E. coli* transformants expressing the *A. facilis* 72W nitrilase, have been used as catalysts to hydrolyze glycolonitrile to glycolic acid at high concentration and in >99% yield [47]. Conversion of acetone cyanohydrin to 2-hydroxyisobutyric acid was also demonstrated using *A. facilis* 72W nitrilase, where subsequent dehydration of 2-hydroxyisobutyric acid produced methacrylic acid [48].

1.2.5 AMIDES VIA NITRILASE CATALYSIS

Nitrilases normally catalyze the addition of two equivalents of water to a nitrile to directly produce the corresponding carboxylic acid and ammonia, but in some instances products other than carboxylic acids have been generated [9]. Inclusion of hydroxylamine (375 mM) in phosphate buffer (pH 7.3) containing benzonitrile (50 mM) and whole cells of *R. rhodochrous* LL100-21 (grown on benzonitrile to induce nitrilase) at 30°C produced a 3% yield of benzohydroxamic acid in addition to benzoic acid; no production of the hydroxamic acid was observed in the absence of the nitrilase catalyst [49]. An enzyme found in the genome of *P. fluorescens* was cloned in *E. coli* and found to have nitrilase activity, and also NHase activity when hydroxycinnamonnitrile was used as the substrate [50]; hydrolysis of this substrate was not due to a combination of NHase and amidase, and a homologous nitrilase from a different strain of *P. fluorescens* was shown to produce only the corresponding acid.

The nitrilase AtNIT1 from the plant *A. thaliana* (overexpressed in *E. coli*) preferred aliphatic nitriles as substrates [51]. Except for fluoro-substituents, substitution at the carbon adjacent to the

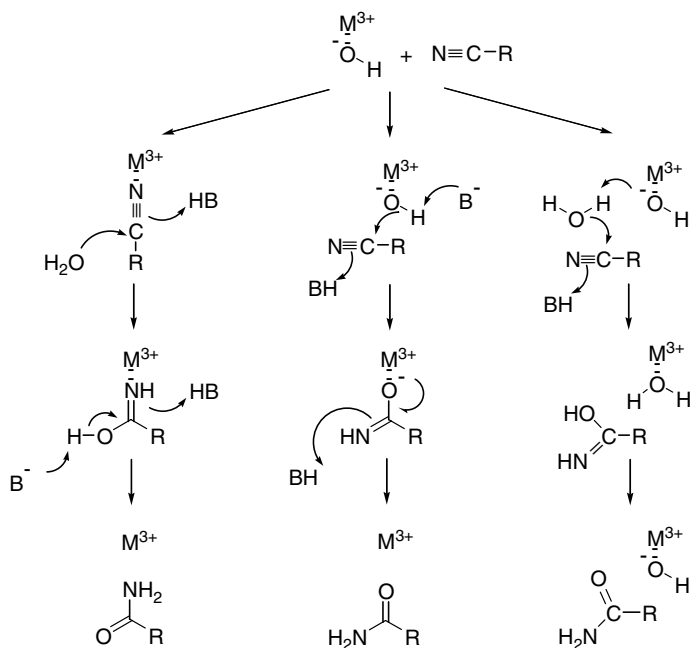
nitrile function completely inhibited hydrolysis. The resolution of (*RS*)-2-fluoroarylacetonitriles by AtNIT1 produced the corresponding (*R*)-amides, and not the expected carboxylic acids, as the major products in 88 to 92% ee at ca. 50% conversion, and recrystallization of the product improved *R*-amide ee to >99%; acid-catalyzed hydrolysis of the (*R*)-amide produced the desired (*R*)-carboxylic acid in 88 to 91% yields without racemization (Scheme 1.10) [52].



SCHEME 1.10 Conversion of 2-fluorobenzyl cyanide to (*R*)-2-fluoro-2-phenylacetamide by AtNIT1 nitrilase.

1.3 NHase and NHase/AMIDASE

There have been several recent reviews on synthetic applications of NHase and NHase/amidase for the conversion of nitriles to amides and carboxylic acids, respectively [53–56], and additional reviews that address the mechanism by which Fe- and Co-containing NHase enzymes convert nitriles to amides [57–62]. The metal ion is believed to function as a Lewis acid, and three reaction mechanisms (Scheme 1.11) have been proposed [57]. For the nonheme Fe-NHase of *Rhodococcus* sp. N771, enzyme activity was found to be regulated by nitrosylation and photo-induced de-nitrosylation of the iron center, and posttranslational modification of two cysteine ligands to cysteine sulphenic acid (Cys-SOH) and cysteine sulphinic acid (Cys-SO₂H) has been proposed as a requirement for catalytic activity [59,60,62,63].



SCHEME 1.11 Proposed mechanism for hydrolysis of nitrile to amide by nitrile hydratase. (Redrawn from Huang, W., Jia, J., Cummings, J., Nelson, M., Schneider, G., and Lindqvist, Y., *Structure*, 5, 691, 1997.)

1.3.1 CRYSTAL STRUCTURES

The crystal structures of an Fe-NHase from *Rhodococcus* sp. N-771 [57,64], and a Co-NHase from *Pseudonocardia thermophila* [65] have been reported. Mutants of *P. thermophila* JCM 3095 NHase that were targeted to produce changes in substrate binding, catalysis, and formation of the active center were prepared, and mutational and structural analyses of the substrate binding and metal specificity of these mutants have also been reported [66]. NHase activity of the wild-type and mutant enzymes were determined for hydration of acrylonitrile, methacrylonitrile, benzonitrile, 3-cyanopyridine, and 4-cyanopyridine. Two α -subunit mutants (T109S and Y114T) were prepared; the T109S mutant had similar characteristics to the wild-type enzyme, whereas the Y114T mutant had a very low cobalt content and catalytic activity. The β -subunit Y68F mutant had an elevated K_m value and a significantly decreased k_{cat} value for acrylonitrile, methacrylonitrile, and benzonitrile.

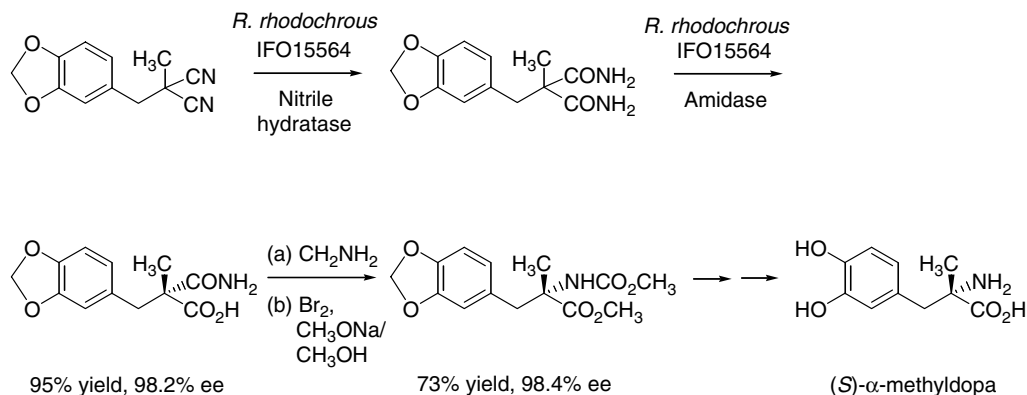
The crystal structure of the cobalt NHase of *Bacillus smithii* SC-J05-1 has been determined [67]. The amino acid sequence identity between the NHase from *B. smithii* and *Rhodococcus* sp. N-771 was 53.2% for the α subunit and 33.9% for the β subunit, and sequence identity between the *B. smithii* and *P. thermophila* NHase was 64.0% for the α subunit and 42.2% for the β subunit. The metal center is located in a central cavity formed at the interface between the *B. smithii* α and β subunits. The ligands to the cobalt atom are three sulfur atoms of Cys120, Cys123, and Cys125, and two main chain amide nitrogen atoms of Ser124 and Cys123; all ligands are in the α subunit. The Phe52 in the β subunit partially covers the metal center and narrows the active site cleft, and it was suggested that this structural feature might contribute to the substrate selectivity of this NHase, where hydrolysis of aliphatic nitriles is preferred to aromatic nitriles.

1.3.2 HETEROLOGOUS PROTEIN EXPRESSION

The sequences flanking NHase genes have been proposed to encode for protein “activators” that transport iron or cobalt to the NHase active site, and are often required for heterologous expression of functional NHase [68]. The heterologous expression of catalytically active *C. testosteroni* NII Fe-NHase in *E. coli* as an $\alpha_2\beta_2$ tetramer was accomplished by co-expression with the *E. coli* GroES and GroEL chaperones [69]. The purified recombinant NHase was highly similar to the enzyme purified from *C. testosteroni*. The mass spectrum of the recombinant enzyme indicated that the majority of the α subunits contained one sulfinic acid modification, and observation of a second sulfinic modification indicated that the expected sulfenic modification was also produced. The production of these different enzyme forms when expressed recombinantly suggested that these posttranslational modifications occurred by auto-oxidation, or were assisted by chaperones or other proteins in *E. coli*.

1.3.3 ENANTIOSELECTIVE NITRILE HYDROLYSIS

A series of α,α -disubstituted malononitriles and related substrates were used to study the substrate specificity and enantioselectivity of the NHase and amidase from *R. rhodochrous* IFO 15564 [70]. The amidase preferentially hydrolyzed the pro-(*R*) carbamyl group of the prochiral diamides produced by the nonenantioselective hydration of the corresponding dinitrile. The introduction of a fluorine atom at the α position caused an inhibitory effect on amidase. A precursor of (*S*)- α -methyldopa was prepared (Scheme 1.12); the substituted malononitrile was first incubated with *R. rhodochrous* cells to produce the (*R*)-amide/carboxylic acid in 95% yield and 98.2% ee, and subsequent methylation of the resulting amide/carboxylic acid with diazomethane, followed by Hofmann rearrangement of the further

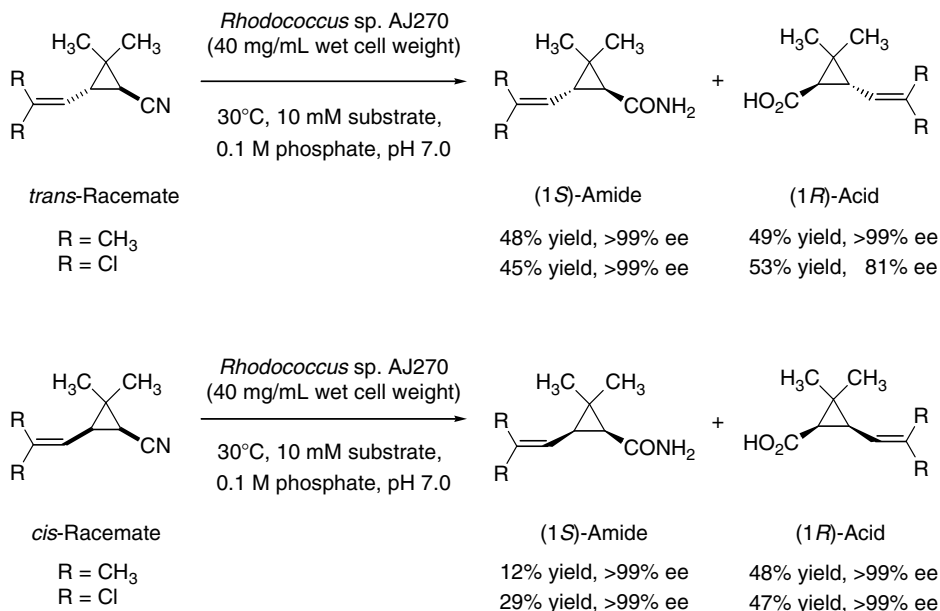


SCHEME 1.12 Enantioselective hydrolysis of the pro-(*R*) carbamyl group of a prochiral diamide produced by the nonenantioselective hydration of a substituted malononitrile.

recrystallized amide/ester (99.8% ee) and trapping with methanol, provided the desired precursor in 73% yield and 98.4% ee. The preparation of (±)-α-cyano-α-fluoro-α-phenylacetic acid (CFPA) from diethyl-α-fluoro-α-phenylmalonate was also demonstrated. α,α-Disubstituted malonamides $RCH_2CMe(CONH_2)_2$ ($R = Ph, 2-ClC_6H_4, 3-ClC_6H_4, 4-ClC_6H_4, 4-MeC_6H_4, 4-MeOC_6H_4, 4-FC_6H_4, 4-BrC_6H_4, PhCH_2, 1\text{-propyl}$) were enantioselectively hydrolyzed by *Rhodococcus* sp. CGMCC 0497 whole cells to produce the corresponding (*R*)-malonamic acids in 92 to 98% yields and 91 to 99% ee [71]; the products could also be converted to (*R*)- or (*S*)-α,α-dialkylated amino acids.

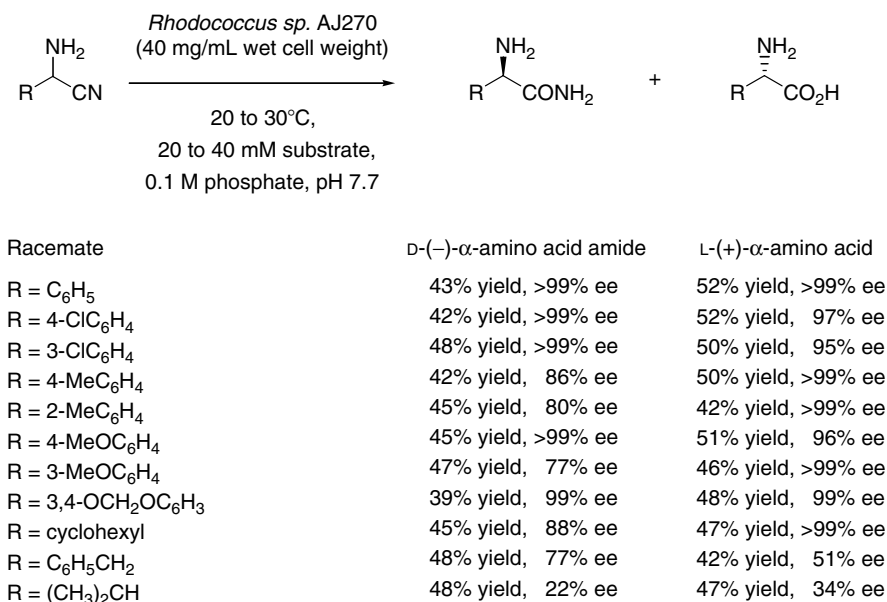
The enantioselective biotransformation of geminally dihalogenated cyclopropanecarbonitriles and amides by *Rhodococcus* sp. AJ270 microbial cells has been reported [72,73]. Both reaction rate and enantioselectivity of the NHase and amidase were strongly controlled by the nature of gem-disubstituents on the cyclopropane ring; the amidase generally exhibited steric dependence on the substituents, whereas both steric and electronic factors affected the NHase. An effective synthesis of optically active 2,2-disubstituted-3-phenylcyclopropanecarboxylic acid and amide in both enantiomeric forms was demonstrated, including a multi-gram scale synthesis of enantiopure 2,2-dichloro-3-phenylcyclopropanecarboxylic acid and amide in both enantiomeric forms. This same microbial catalyst was used to examine the hydrolysis of 2,2-dimethyl-3-substituted cyclopropanecarbonitriles [74]. *Cis*-3-aryl-2,2-dimethylcyclopropanecarbonitriles were not hydrolyzed, but racemic *trans*-isomers were hydrolyzed in high yields and with high enantioselectivity (in most instances) to produce (+)-(1*R*,3*R*)-3-aryl-2,2-dimethylcyclopropanecarboxylic acids and (−)-(1*S*,3*S*)-3-aryl-2,2-dimethylcyclopropanecarboxamides; optically pure geminally dimethyl-substituted cyclopropanecarboxylic acids and amides, including chrysanthemic acids, were produced in both enantiomeric forms (Scheme 1.13).

Chiral α-substituted α-amino acids have found use in the preparation of pharmaceuticals, and numerous methods have been reported for their preparation. *Rhodococcus* sp. AJ270 cells catalyzed the enantioselective hydrolysis of α-substituted DL-glycine nitriles to produce D-(−)-α-amino acid amides and L-(+)-α-amino acids in high yields and ee (Scheme 1.14) [75]. The enantioselective synthesis of (*R*)-(+)-α-arylalanine amides and (*S*)-(−)-α-arylalanines was also accomplished by the kinetic resolution of racemic amides using the amidase activity of *Rhodococcus* sp. AJ270 whole cells [76]. Both the reaction rate and *S*-enantioselectivity of the biocatalytic kinetic resolution were dependent upon the nature and the substitution pattern of the aryl substituent. Racemic α-ethyl phenylglycine amide was

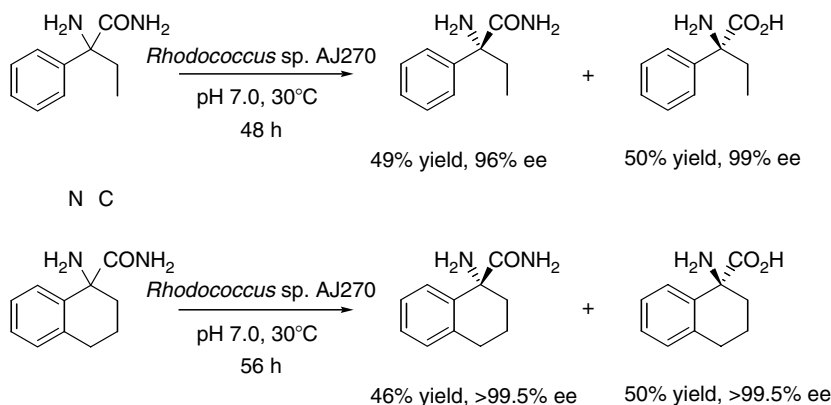


SCHEME 1.13 Biocatalytic hydration/hydrolysis of racemic chrysanthemic nitriles.

efficiently resolved into (*R*)-(+)- α -ethyl phenylglycine amide (96% ee) and (*S*)-(–)- α -ethyl phenylglycine (99% ee), and (*R*)-(+)-1-amino-1-carbamoyl-1,2,3,4-tetrahydronaphthalene and (*S*)-(–)-1-amino-1-carboxy-1,2,3,4-tetrahydronaphthalene were prepared in excellent yields at >99.5% ee (Scheme 1.15). The chemical hydrolysis of the remaining chiral amide could also be performed with no measurable loss of chirality, making both enantiomeric



SCHEME 1.14 Enantioselective biotransformations of DL- α -amino nitriles.



SCHEME 1.15 Biocatalytic kinetic resolution of racemic α -ethyl phenylglycine amide and 1-amino-1-carbamoyl-1,2,3,4-tetrahydronaphthalene.

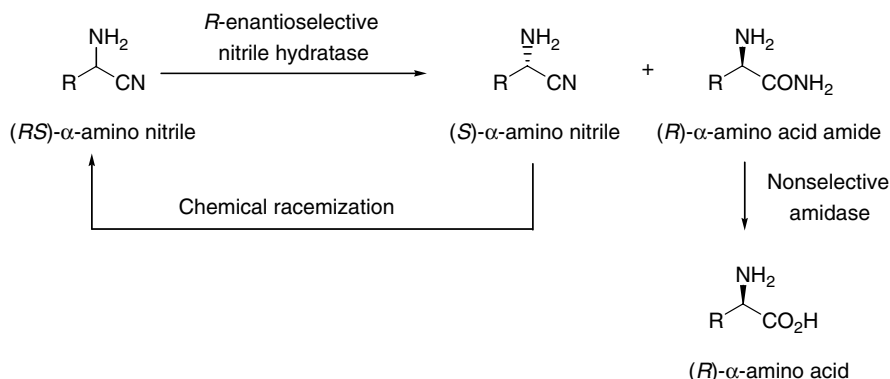
forms of the arylalanines readily available from the racemic amides. *Rhodococcus* sp. AJ270 was used to prepare enantiopure *S*-(+)-2-aryl-3-methylbutyric acids and *R*-(+)-2-aryl-3-methylbutyramides from the hydrolysis of 2-aryl-3-methylbutyronitriles [77]; in this application, the NHase displayed a low *S*-enantioselectivity for the nitriles, whereas the amidase had a strict *S*-enantioselectivity for the 2-aryl-3-methylbutyramides.

Five new bacterial isolates, *R. erythropolis* 11.1, *Rhodococcus* sp. 27.1, *Pantoea endophytica* 26.2.2, *Pantoea* sp. 17.3.1, and *Nocardioides* sp. 29.3, were each found to have an enantioselective NHase for hydration of (*RS*)-2-phenylpropionitrile and (*RS*)-phenylglycine nitrile [78]. The NHase enantioselectivities were generally low and poorly (*S*)-selective. The amidases were either (*S*)- or (*R*)-enantioselective, and could be used in kinetically controlled reactions for synthesis of pure (*S*)- or pure (*R*)-phenylglycine, respectively. *P. endophytica* produced (*S*)-phenylglycine (>99% ee) through hydrolysis of (*S*)-phenylglycine amide by an (*S*)-specific amidase, and (*R*)-phenylglycine (>99% ee) was produced using the (*R*)-selective amidase of *Pantoea* sp.

A recent patent application disclosed a method for the production of artificially evolved *R*- or *S*-enantioselective NHases by recombining two or more genes encoding a nonenantioselective NHase and/or by mutating one or more nonenantioselective NHase genes in one or more cycles of recombination or mutation. The recombination of two or more NHase genes was typically performed by recursive combination, whole genome recombination, synthetic recombination, or *in silico* recombination [79]. This method could also be used to produce enantioselective nitrilases. An enantioselective method for the conversion of a racemic mixture of aminonitriles to *R*-amino acids (Scheme 1.16) or *S*-amino acids was described.

DL-Methionine has been prepared from 2-amino-4-methylthiobutyronitrile or the corresponding amide at 35 to 60°C with a *P. thermophila* JC M3095 or JC M3032 catalyst that produced both NHase and amidase [80]; in this case the combination of NHase and amidase activities afforded no enantioselectivity, and the product was produced as the racemate. The combination of NHase and amidase activities of *R. rhodochrous* DSM 43198 was also used to convert 2-hydroxy-4-methylthiobutanenitrile to DL-methionine at 5 to 40°C [81].

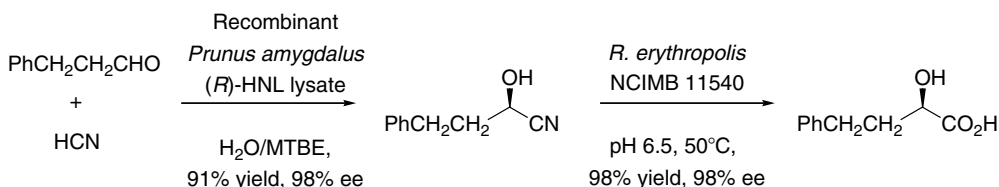
Chiral α -hydroxycarboxylic acids have been prepared by hydrolysis of (*R*)- or (*S*)-cyano-hydrins to the corresponding (*R*)- or (*S*)- α -hydroxycarboxylic acids using whole cells of *R. erythropolis* NCIMB 11540 [82,83] that have a NHase/amidase enzyme combination with high specific activity for hydrolysis of the nitrile group of cyanohydrins. Enantiopure cyanohydrins



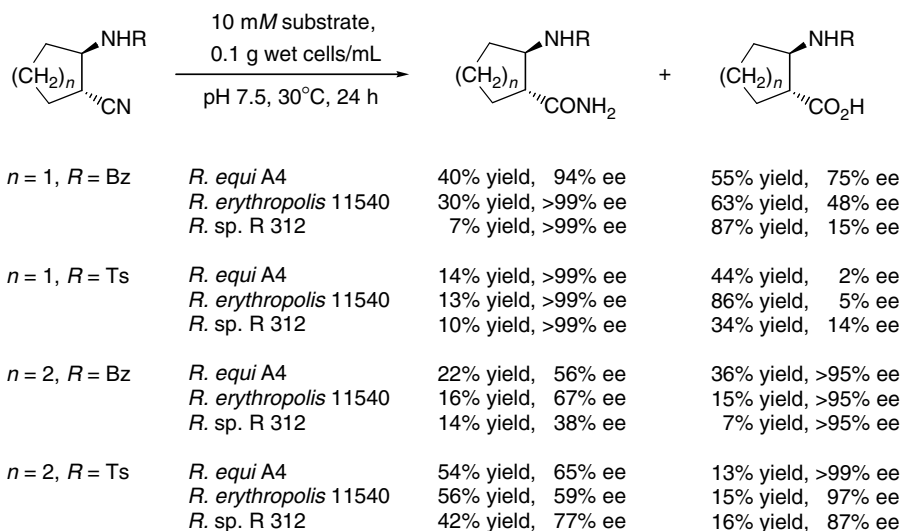
SCHEME 1.16 Dynamic resolution of DL-α-amino nitriles using an enantioselective nitrile hydratase.

were readily prepared using (*R*)- or (*S*)-hydroxynitrile lyases (HNL) [84,85], and the products were obtained in high yield without racemization, decomposition, or side reactions. The pharmaceutical intermediate (*R*)-2-hydroxy-4-phenylbutyric acid was prepared using 13 g/L of the corresponding nitrile in 98% yield (ee > 98%) (Scheme 1.17). The NHase and amidase from *R. erythropolis* NCIMB 11540 were separately cloned and expressed in *E. coli* [86], and cell extracts were used for the hydrolysis of different aromatic cyanohydrins. The production of either α-hydroxy amides or acids was demonstrated with retention of enantiopurity, and the production of NHase and amidase in separate transformants allowed for the use of additional amidase catalyst in instances where amide hydrolysis was rate-limiting.

The effect of the *N*-protecting group on the biotransformation of *N*-tolylsulfonyl- and *N*-butyloxycarbonyl-protected β-amino nitriles to the corresponding β-amino amides and acids was examined using whole cells of *Rhodococcus* sp. R312 or *R. erythropolis* NCIMB 11540 as catalysts [87,88]. The bioconversion products of five-membered carbocyclic nitriles were mainly the respective acids, whereas the six-membered carbocyclic nitriles produced amides as the major reaction product. The type of protecting group (sulfonamide or carbamate) did not affect the nature of the products produced, but yields were higher using the carbamate-protected derivatives. The enantioselectivity of the NHase/amidase activities of *R. equi* A4, *R. erythropolis* NCIMB 11540, and *Rhodococcus* sp. R312 was evaluated for conversion of *cis*- and *trans*-2-aminocyclopentane/cyclohexane nitriles to the corresponding β-amino amides and acids (Scheme 1.18) [89]. Five-membered alicyclic 2-amino nitriles were converted significantly faster than the six-membered ring compounds, and the products of *trans*-2-amino nitriles (amides and acids) were produced considerably faster than the *cis*-counterparts (which produced only amides). The ees of the *cis* isomers were consistently lower than that of the *trans* isomers.



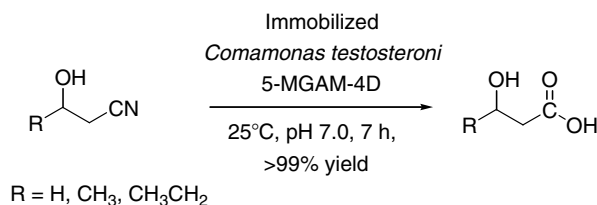
SCHEME 1.17 Preparation of (*R*)-2-hydroxy-4-phenylbutyric acid using a combination of an (*R*)-hydroxynitrile lyase, nitrile hydratase, and amidase.



SCHEME 1.18 Enantioselective conversion of *cis*- and *trans*-2-aminocyclopentane/cyclohexane nitriles to the corresponding β -amino amides and acids.

1.3.4 NHase/AMIDASE CATALYSTS FOR PRODUCTION OF ACHIRAL CARBOXYLIC ACIDS

Microbial catalysts having a combination of NHase and amidase activities had a significantly higher specific activity than the microbial nitrilases that were screened for hydrolysis of 3-hydroxyalkanenitriles to the corresponding 3-hydroxyalkanoic acids (Scheme 1.19) [90]. *C. testosteroni* 5-MGAM-4D cells were immobilized in alginate beads, and the resulting NHase/amidase biocatalyst hydrolyzed 3-hydroxyvaleronitrile to 3-hydroxyvaleric acid in 99 to 100% yields in a series of 85 consecutive batch reactions with biocatalyst recycle for the production of 118 g/L 3-hydroxyvaleric acid. The catalyst productivity for this series of reactions was 670 g 3-hydroxyvaleric acid/g dry cell weight, the initial volumetric productivity was 44 g 3-HVA/L/h, and the recovered NHase and amidase activities in the final reaction were 29% and 40%, respectively, of their initial activities. Similar results were obtained for hydrolysis of 3-hydroxybutyronitrile and 3-hydroxypropionitrile. The combined NHase and amidase activities of *C. testosteroni* 5-MGAM-4D have also been employed as catalyst for the hydrolysis of acrylonitrile and methacrylonitrile to acrylic acid and methacrylic acid, respectively [91], and for hydrolysis of acetone cyanohydrin to 2-hydroxyisobutyric acid [92].

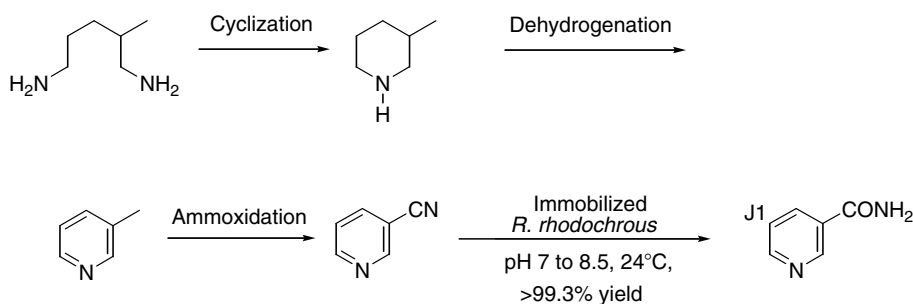


SCHEME 1.19 Two-step conversion of 3-hydroxyalkanenitriles to 3-hydroxyalkanoic acids using nitrile hydratase/amidase.

1.3.5 COMMERCIALIZED PROCESSES USING NHASE CATALYSTS

1.3.5.1 Nicotinamide (Niacinamide)

Lonza Guangzhou Fine Chemicals manufactures nicotinamide by a process where 2-methyl-1,5-diaminopentane (a by-product of nylon-6,6 manufacture) is first converted to 3-cyanopyridine in a series of three chemically catalyzed reactions, then the nitrile is hydrated to nicotinamide using *R. rhodochrous* J1 cells immobilized in polyacrylamide particles (Scheme 1.20) [93–95]. A continuous feed of 3-cyanopyridine at concentrations of between 10 and 20 wt.% is supplied in the direction of process flow, with a countercurrent feed of biocatalyst in a series of stirred-tank batch reactors. The process generates >3500 MT of nicotinamide/y with >99.3% selectivity at 100% conversion of 3-cyanopyridine. An additional plant is planned, with an initial 6000 MT/y capacity that could be expanded to 9000 MT/y [96].



SCHEME 1.20 Process for production of nicotinamide employing biocatalytic hydration of 3-cyanopyridine.

R. rhodochrous J1 has a high K_m (200 mM) and low tolerance for 3-cyanopyridine, and contains a red pigment that requires decolorizing the product solution. Recent work by Lonza has focused on developing alternate biocatalysts for nicotinamide production, including *Amycolatopsis*, *Actinomadura*, and *Rhodococcus* [97–99]. *Rhodococcus* sp. FZ4 (DSM 13597) was isolated from soil samples using 3-pyridinaldoxime as inducer of NHase activity [96]; it expressed a cobalt-dependent NHase with improved properties for production of nicotinamide relative to J1 (Table 1.2). Using unimmobilized FZ4 cells, a total of 1876 g nicotinamide/g FZ4 dry cell weight was obtained in a single batch reaction to produce ~60% (w/v) nicotinamide in 0.1 M potassium phosphate buffer (pH 6.0).

1.3.5.2 5-Cyanovaleramide

A biocatalytic process was commercialized for production of 5-cyanovaleramide (5-CVAM), a starting material in the manufacture of the herbicide azafenidin (Scheme 1.21) [100]. NHase-catalyzed production of 5-CVAM resulted in higher yields of 5-CVAM, higher catalyst productivity, lower by-product production, and generated significantly less process waste than alternative chemical methods. The process was first piloted using *R. erythropolis* A4 cells [101–103] immobilized in calcium alginate beads. Reactions were run at 5°C, as the stability of the enzyme decreased markedly above this temperature. Sixty consecutive batch reactions converted a total of 1.1 MT of adiponitrile to produce a 9.0 wt.% solution of 5-CVAM in 93% yield, with a catalyst productivity of greater than 1000 kg 5-CVAM/kg dry cell weight.

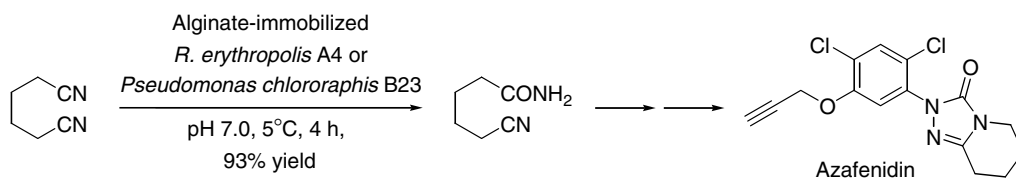
TABLE 1.2
Comparison of *Rhodococcus* sp. FZ4 and *R. rhodochrous* J1 for Hydration of 3-Cyanopyridine

	<i>Rhodococcus</i> sp. FZ4	<i>R. rhodochrous</i> J1
K_m (mM 3-cyanopyridine)	160	200
Relative activity (%) in 10% (w/v) 3-cyanopyridine	100	63
Relative activity (%) in 30% (w/v) nicotinamide	100	n.d.
Relative activity after incubation at 60°C for 15 min	93	80
Pigmentation	None	red

n.d., not determined.

5-CVAM was isolated by distillation of water, dissolution of the resulting solids in boiling methanol, and filtration of insoluble adipamide and salts; the resulting methanolic 5-CVAM solution was used directly in the subsequent azafenidin process step.

Commercial-scale fermentation of *R. erythropolis* A4 required a light-activation step of the microbial NHase [104,105] as an additional cost for catalyst manufacture. Further screening of microbial NHases identified *P. chlororaphis* B23 as a more effective biocatalyst for 5-CVAM production. *P. chlororaphis* B23 was first isolated and characterized by H. Yamada and coworkers [106–108], and was later used by the Nitto Chemical Industry Co. for the manufacture of acrylamide from acrylonitrile [109–112]. In the initial commercial-scale production run, *P. chlororaphis* B23 microbial cells were immobilized in calcium alginate beads, and 58 consecutive batch reactions were run at 5°C with biocatalyst recycle to convert a total of 12.7 MT of adiponitrile to 13.6 MT of 5-CVAM (97% conversion, 96% selectivity, 93% yield) as a 19.2 wt.% solution in water [113,114]. The immobilized-cell catalyst was highly regioselective, with less than 5% selectivity to by-product adipamide. The catalyst productivity in this first commercial-scale run was 3150 kg 5-CVAM/kg dry cell weight; a total of ~150 MT of 5-CVAM were prepared by this process for azafenidin manufacture.



SCHEME 1.21 Regioselective biocatalytic hydration of adiponitrile to 5-cyanovaleramide.

1.3.5.3 Acrylamide

New NHase catalysts continue to be developed and compared with the existing wild-type whole-cell catalysts used in the commercial production of acrylamide from acrylonitrile. Mitsubishi Rayon has produced mutant enzymes of *R. rhodochrous* J1 NHase with significantly improved thermal stability and catalytic activity at 50 to 70°C [115,116]. SNF Floerger (Saint-Etienne, France) has licensed and commercialized the manufacture of acrylamide using Mitsubishi Rayon's immobilized *R. rhodochrous* J1 [117], and has independently developed a *R. pyridinovorans* whole-cell catalyst for this process [118]. A thermostable NHase has been

cloned from *Geobacillus thermoglucosidasius* strain Q-6 and expressed in *E. coli* [119]; this catalyst had good performance at high acrylonitrile (6 wt.%) and acrylamide (35 wt.%) concentrations, and the enzyme retained >35% initial activity after 30 min at 70°C. Novel mutants of *P. thermophila* JCM3095 NHase have been produced, where substitutions at three positions in the α subunit and 15 positions in the β subunit resulted in mutant enzymes with improved specific activity or substrate selectivity [120].

An *E. coli* transformant that expressed a thermally stable NHase produced by *C. testosteroni* 5-MGAM-4D was immobilized in calcium alginate and evaluated for production of acrylamide in batch reactions with catalyst recycle [121,122]. Catalyst productivity decreased with increasing acrylonitrile concentration or reaction temperature, but was relatively insensitive to acrylamide concentration. A total of 975 g acrylamide/g dry cell weight and 89 g acrylamide/L were produced at 5°C in 206 consecutive batch reactions with catalyst recycle, with initial and final volumetric productivities of 142 and 76 g acrylamide/L/h, respectively. This catalyst productivity was comparable to that of commercial polyacrylamide-immobilized *Rhodococcus* sp. 774 and *P. putida* B23 catalysts (500 and 850 g acrylamide/g dry cell weight, respectively), but less than that of the currently employed polyacrylamide-immobilized *R. rhodochrous* J1 (>7000 g acrylamide/g dry cell weight) [123]. Although the 5-MGAM-4D NHase had good thermal stability at 35°C, catalyst productivity at this temperature was significantly less than that at 5°C. The major determinant of biocatalyst productivity was the concentration of acrylonitrile in the reaction mixture, as was the case in previous studies with thermally stable microbial NHases, where the nucleophilic reaction of protein functional groups with acrylonitrile was proposed as a mechanism for enzyme inactivation [124,125].

In addition to the commercial production of >30,000 MT/y of acrylamide using *R. rhodochrous* J1, >15,000 MT acrylamide/y are currently produced in the Peoples Republic of China (PRC) using *Nocardia* sp. microbial NHase biocatalysts. In an initial study of the suitability of *Nocardia* sp. 9112-118 for commercial production of acrylamide, the cells were immobilized in alginate and used to produce 250 g/L acrylamide at 20 to 23°C in a 5000 L batch reactor [126]. For the fermentation of *Nocardia* sp. RS, optimization of pH regulation and addition of glucose- Co^{2+} in fed-batch fermentations increased NHase activity to 10,195 U/mL fermentation broth, among the highest volumetric productivities for all reported NHase fermentations [127]. The acrylamide tolerance of *Nocardia* sp. RS was improved by feeding acrylonitrile periodically into shake flasks containing growing cells and screening of cells surviving in the high concentration of the resulting acrylamide, thereby producing the acrylamide-tolerant *Nocardia* sp. strain RS-1 [128,129]. The final acrylamide concentration and percent conversion of acrylonitrile catalyzed by the RS-1 strain were 587 g/L and 99.97%, respectively, a 30.6% higher final acrylamide concentration than that produced by the parent RS strain; further optimization of reaction conditions using RS-1 produced a final acrylamide concentration of 641 g/L. Recently, two NHase genes, NHBA and NHBAX, from *Nocardia* sp. YS-2002 have been cloned, and expressed in *E. coli* [130].

Alternatives to the polyacrylamide immobilization of microbial cell catalysts for industrial acrylamide production have also been evaluated. A hollow-fiber membrane bioreactor was evaluated for the conversion of acrylonitrile to acrylamide using unimmobilized cells of *Nocardia* sp., a microbial cell catalyst that is used for commercial production of acrylamide by the Nantian Corporation (Jiangsu, PRC) [131]. Initial studies at laboratory scale used a 10% (v/v) fermentation broth as a catalyst charge at 20°C. Different membrane materials were evaluated, including polysulfone (PS), polyvinylidene difluoride (PVDF), and polyacrylonitrile (PAN). The PS hollow-fiber membrane was scaled up, having the advantages of high flux, high resistance to fouling, operation in a cross-flow filtration mode, and easy cleanup. A 50,000-Da nominal molecule weight cutoff was preferred for protein filtration. An initial decay of flux, almost 35% of the initial filtration rate, occurred during the first 2 to 3 h of the

filtration, after which the filtration rate remained unchanged; the decay of the membrane flux was attributed to absorption and compaction of cells and protein on the inner surface of the hollow fibers. The conversion rate of acrylonitrile was 99.9%, and the productivity of acrylamide was 20.67 g/g cell/h over 5 h of operation. The reaction was scaled up in a 6000 L hollow-fiber membrane reactor to produce 300 g/L acrylamide over 17 h. The conversion rate of acrylonitrile was 99.9%, and the efficiency of enzyme activity and acrylamide productivity were 10.96 g/g cell/h and 187.2 g/g cell, respectively, compared to 5.22 g/g cell/h and 112 g/g cell, respectively, when using the immobilized-cell catalyst in a fed-batch mode over 20 h.

A process has been described for producing amides, including acrylamide, that employs unimmobilized microbial cells at a reaction temperature of 10°C, where the cells have an NHase specific activity of at least 50 U/mg dry cell weight [132]. This process claims the advantage of efficiently producing acrylamide without experiencing a loss of catalyst specific activity as a result of immobilization, and increased reaction rates and volumetric productivity were observed when comparing unimmobilized cells with an equivalent amount (on a dry cell weight basis) of immobilized cells.

In a study comparing free-cell and immobilized-cell catalysts, *R. rhodochrous* M33 cells were immobilized in acrylamide-based polymer gels, and compared to unimmobilized cells for the production of acrylamide [133]. The optimum pH and temperature for NHase activity in both free and immobilized cells were 7.4 and 45°C, respectively, but the optimum temperature for acrylamide production was 20°C. The immobilized-cell NHase was more stable than the free-cell NHase in the presence of high concentrations of acrylamide. Under optimal conditions, a final acrylamide concentration of ~400 g/L was achieved, with a conversion yield of almost 100% after 8 h of reaction when using 150 g/L of immobilized cells (corresponding to a 1.91 g dry cell weight/L); the immobilized cell enzyme activity decreased rapidly with repeated use. Comparing the quality of the acrylamide produced by immobilized cells with that produced by free cells, the aqueous acrylamide solution produced by the former had lower color, salt content, turbidity, and foam formation than that produced by the latter; this aqueous acrylamide solution could be used directly in commercial applications, whereas the free-cell product required further downstream purification.

1.3.6 MODIFICATION OF NITRILE-CONTAINING POLYMERS

Enzymatic treatment of PAN polymers by NHase, or a combination of NHase and amidase, can be used to hydrate and/or hydrolyze surface nitrile groups; the treated polymers or fibers were more hydrophilic, had improved antistatic properties, and readily reacted with acid dyes under conditions where untreated polymers were inert. *R. rhodochrous* NCIMB 11216 produced NHase and amidase activities that hydrolyzed nitrile groups of both granular PAN and acrylic fibers [134]; 1.8% of PAN40 (molecular mass, 40 kDa) total available nitrile groups and 1.0% of PAN190 (molecular mass, 190 kDa) total available nitrile groups were converted to the corresponding carboxylic acids, and a maximum of 16% of surface nitrile groups of acrylic fibers were converted only to the corresponding amides. Similar improvements in acid dyeing of acrylic fibers was achieved by treatment with the NHase of *Arthrobacter* sp. Ecu1101 [135], or *Brevibacterium imperiale* and *Corynebacterium nitrilophilus* [136].

1.3.7 NITRILASE OR NHASE?

Bacterial nitrile hydrolysis has been widely documented, whereas there are relatively few reports of fungal nitrile hydrolysis. A nitrile-converting enzyme activity was induced in *Aspergillus niger* K10 by 3-cyanopyridine [137], and the resulting microbial biocatalyst hydrolyzed the cyano group of benzonitrile, 3- and 4-substituted benzonitrile, cyanopyridines,

2-phenylacetonitrile, and thiophen-2-acetonitrile into acid and/or amide groups. Amides were significant reaction products for hydrolysis of 2- and 4-cyanopyridine, 4-chlorobenzonitrile, 4-tolunitrile, and 1,4-dicyanobenzene, while α -substituted acrylonitriles were converted only to amides. This pattern of nitrile conversion is novel for filamentous fungi that hydrolyze nitriles mostly through the nitrilase pathway, therefore the nitrile-converting enzyme is either a nitrilase affording extremely large amounts of some amides or a NHase.

1.4 CONCLUSIONS

Both nitrilases and NHases have found increasing use in the production of agrochemicals and pharmaceuticals, as well as commodity chemicals. The advent of methods for directed evolution of enzymes has made it possible to move beyond the screening of microbial culture collections for the desired catalyst activity; enzymes can be evolved for improvement in enantioselectivity and regioselectivity, temperature stability, substrate K_m , and substrate and product inhibition or inactivation. The expression of heterologous nitrilases and NHases in suitable hosts often results in significant increases in microbial specific activity when compared with that of the wild-type microbe from which the gene was isolated. It is interesting to note that for both nitrilases and NHases, microbial cell catalysts are utilized much more frequently than isolated enzymes, as there are rarely any microbially catalyzed reactions that compete with these enzymes for substrate, or produce undesired reaction by-products.

Many of the recent examples cited above demonstrate the use of nitrilase and NHase as catalysts to generate product concentrations that are required for economical commercial production, and report volumetric productivity (g product/L/h) and biocatalyst productivity (g product/g microbial biocatalyst dry cell weight) in these reactions. It is now generally recognized that catalyst cost and overall process economics must be considered in the design of any industrial process employing a biocatalyst, where the cost of manufacture must favorably compete with alternative chemical methods. The design of such processes must take into account the expected capital costs and downstream processing costs (e.g., conversion of the ammonium salt of a carboxylic acid to the free acid, separation and recovery of product from reaction by-products in high yield and purity, disposal of reaction by-products and salts generated in product isolation/purification), and a number of recent publications and patents address the engineering aspects of scale-up along with biocatalyst development.

REFERENCES

1. O'Reilly, C. and Turner, P.D., The nitrilase family of CN hydrolyzing enzymes — a comparative study, *J. Appl. Microbiol.*, 95, 1161, 2003.
2. Martinkova, L. and Mylerova, V., Synthetic applications of nitrile-converting enzymes, *Curr. Org. Chem.*, 7, 1279, 2003.
3. Martinkova, L. and Kren, V., Nitrile- and amide-converting microbial enzymes: stereo-, regio- and chemoselectivity, *Biocatal. Biotransform.*, 20, 73, 2002.
4. Banerjee, A., Sharma, R., and Banerjee, U.C., The nitrile-degrading enzymes: current status and future prospects, *Appl. Microbiol. Biotechnol.*, 60, 33, 2002.
5. Brenner, C., Catalysis in the nitrilase superfamily, *Curr. Opin. Struct. Biol.*, 12, 775, 2002.
6. Groger, H., Enzymatic routes to enantiomerically pure aromatic α -hydroxy carboxylic acids: a further example for the diversity of biocatalysis, *Adv. Synth. Catal.*, 343, 547, 2001.
7. Wieser, M. and Nagasawa, T., Stereoselective nitrile-converting enzymes, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, chap. 17.

8. Pace, H.C. and Brenner, C., The nitrilase superfamily: classification, structure and function, *Genome Biol.* (online computer file), 2, reviews 0001.1, 2001.
9. Kobayashi, M., Goda, M., and Shimizu, S., Nitrilase catalyzes amide hydrolysis as well as nitrile hydrolysis. *Biochem. Biophys. Res. Commun.*, 253, 662, 1998.
10. Ress-Loeschke, M., Hauer, B., Mattes, R., and Engels, D., U.S. Patent Appl. 2003157672 A1, 2003.
11. Herai, S., Hashimoto, Y., Higashibata, H., Maseda, H., Ikeda, H., Omura, S., and Kobayashi, M., Hyper-inducible expression system for *Streptomyces*. *Proc. Natl. Acad. Sci. USA*, 101, 14031, 2004.
12. Chauhan, S., DiCosimo, R., Fallon, R.D., Gavagan, J.E., and Payne, M.S., U.S. Patent 6,870,038 B2, 2005.
13. Heinemann, U., Engels, D., Buerger, S., Kiziak, C., Mattes, R., and Stolz, A., Cloning of a nitrilase gene from the cyanobacterium *Synechocystis* sp. strain PCC6803 and heterologous expression and characterization of the encoded protein, *Appl. Environ. Microbiol.*, 69, 4359, 2003.
14. Park, W.J., Kriechbaumer, V., Mueller, A., Piotrowski, M., Meeley, R.B., Gierl, A., and Glawischnig, E., The nitrilase ZmNIT2 converts indole-3-acetonitrile to indole-3-acetic acid, *Plant Physiol.*, 133, 794, 2003.
15. DeSantis, G., Wong, K., Farwell, B., Chatman, K., Zhu, Z., Tomlinson, G., Huang, H., Tan, X., Bibbs, L., Chen, P., Kretz, K., and Burk, M.J., Creation of a productive, highly enantioselective nitrilase through gene site saturation mutagenesis (GSSM), *J. Am. Chem. Soc.*, 125, 11476, 2003.
16. DeSantis, G., Zhu, Z., Greenberg, W.A., Wong, K., Chaplin, J., Hanson, S.R., Farwell, B., Nicholson, L.W., Rand, C.L., Weiner, D.P., Robertson, D.E., and Burk, M.J., An enzyme library approach to biocatalysis: development of nitrilases for enantioselective production of carboxylic acid derivatives, *J. Am. Chem. Soc.*, 124, 9024, 2002.
17. Robertson, D.E., Chaplin, J.A., DeSantis, G., Podar, M., Madden, M., Chi, E., Richardson, T., Milan, A., Miller, M., Weiner, D.P., Wong, K., McQuaid, J., Farwell, B., Preston, L.A., Tan, X., Snead, M.A., Keller, M., Mathur, E., Kretz, P.L., Burk, M.J., and Short, J.M., Exploring nitrilase sequence space for enantioselective catalysis, *Appl. Environ. Microbiol.*, 70, 2429, 2004.
18. Squires, C. and Talbot, H., *Pseudomonas fluorescens*: a robust manufacturing platform, *Speciality Chemicals Magazine*, 24, 25, 2004.
19. Kaul, P., Banerjee, A., Mayilraj, S., and Banerjee, U.C., Screening for enantioselective nitrilases: kinetic resolution of racemic mandelonitrile to (*R*)-(-)-mandelic acid by new bacterial isolates, *Tetrahedron Asymmetry*, 15, 207, 2004.
20. Favre-Bulle, O., Pierrard, J., David, C., Morel, P., and Horbez, D., U.S. Patent 6180359 B1, 2001.
21. Rey, P., Rossi, J.-C., Taillades, J., Gros, G., and Nore, O., Hydrolysis of nitriles using an immobilized nitrilase: applications to the synthesis of methionine hydroxy analogue derivatives, *J. Agric. Food Chem.*, 52, 8155, 2004.
22. Favre-Bulle, O. and Le Thiesse, J.-C., U.S. Patent Appl. 2004082046 A1, 2004.
23. Pierrard, J., Favre-Bulle, O., and Jourdat, C., PCT Int. Appl. WO 2001034786 A1, 2001.
24. Nagasawa, T. and Matsuyama, A., Jpn. Kokai Tokyo Koho JP 2001054380 A2, 2001.
25. Effenberger, F., Forster, S., and Wajant, H., Hydroxymitrile lyases in stereoselective catalysis, *Curr. Opin. Biotechnol.*, 11, 532, 2000.
26. Heinemann, U., Kiziak, C., Zibek, S., Layh, N., Schmidt, M., Griengl, H., and Stolz, A., Conversion of aliphatic 2-acetoxynitriles by nitrile-hydrolysing bacteria, *Appl. Microbiol. Biotechnol.*, 63, 274, 2003.
27. Chaplin, J.A., Levin, M.D., Morgan, B., Farid, N., Li, J., Zhu, Z., McQuaid, J., Nicholson, L.W., Rand, C.A., and Burk, M.J., Chemoenzymatic approaches to the dynamic kinetic asymmetric synthesis of aromatic amino acids, *Tetrahedron Asymmetry*, 15, 2793, 2004.
28. Zelinski, T., Kessler, M., Hauer, B., and Friedrich, T., PCT Int. Appl. WO 2004076655 A1, 2004.
29. Bensoussan, C., Bontoux, M.C., Gelo, P.M., and Foray, F., Fr. Demande 2822460 A1, 2002.
30. Cooling, F.B., Fager, S.K., Fallon, R.D., Folsom, P.W., Gallagher, F.G., Gavagan, J.E., Hann, E.C., Herkes, F.E., Phillips, R.L., Sigmund, A., Wagner, L.W., Wu, W., and DiCosimo, R., Chemoenzymatic production of 1,5-dimethyl-2-piperidone, *J. Mol. Catal. B Enzym.*, 11, 295, 2001.
31. Hann, E.C., Sigmund, A.E., Hennessey, S.M., Gavagan, J.E., Short, D.R., Ben-Bassat, A., Chauhan, S., Fallon, R.D., Payne, M.S., and DiCosimo, R., Optimization of an immobilized-cell biocatalyst for production of 4-cyanopentanoic acid, *Org. Process Res. Dev.*, 6, 492, 2002.

32. Chauhan, S., Wu, S., Blumberman, S., Fallon, R.D., Gavagan, J.E., DiCosimo, R., and Payne, M.S., Purification, cloning, sequencing and over-expression in *Escherichia coli* of a regioselective aliphatic nitrilase from *Acidovorax facilis* 72W, *Appl. Microbiol. Biotechnol.*, 61, 118, 2003.
33. Jennings, R.A., Johnson, D.R., Seamans, R.E., and Zeller, J.R., U.S. Patent 5,362,883, 1994.
34. Burns, M.P. and Wong, J.W., U.S. Patent Appl. 2005009154 A1, 2005.
35. Effenberger, F. and Osswald, S., Enzyme-catalyzed reactions. 41. Selective hydrolysis of aliphatic dinitriles to monocarboxylic acids by a nitrilase from *Arabidopsis thaliana*, *Synthesis*, 12, 1866, 2001.
36. Kamachi, H. and Aoki, H., Jpn. Kokai Tokkyo Koho JP 2001136977 A2, 2001.
37. Fujita, I., Aoki, H., and Wada, K., Jpn. Kokai Tokkyo Koho JP 2003325194 A2, 2003.
38. Dadd, M.R., Claridge, T.D.W., Walton, R., Pettman, A.J., and Knowles, C.J., Regioselective biotransformation of the dinitrile compounds 2-, 3- and 4-(cyanomethyl)benzonitrile by the soil bacterium *Rhodococcus rhodochrous* LL100-21, *Enzyme Microb. Technol.*, 29, 20, 2001.
39. Effenberger, F. and Osswald, S., (*E*)-Selective hydrolysis of (*E,Z*)- α,β -unsaturated nitriles by the recombinant nitrilase AtNIT1 from *Arabidopsis thaliana*, *Tetrahedron Asymmetry*, 12, 2581, 2001.
40. Hann, E.C., Sigmund, A.E., Fager, S.K., Cooling, F.B., Gavagan, J.E., Ben-Bassat, A., Chauhan, S., Payne, M.S., and DiCosimo, R., Regioselective biocatalytic hydrolysis of (*E,Z*)-2-methyl-2-butene-nitrile for production of (*E*)-2-methyl-2-butenic acid, *Tetrahedron*, 60, 577, 2004.
41. Chauhan, S., DiCosimo, R., Fallon, R.D., Gavagan, J.E., and Payne, M.S., U.S. Patent 6455730 B1, 2002.
42. Brady, D., Beeton, A., Zeevaart, J., Kgaje, C., van Rantwijk, F., and Sheldon, R.A., Characterisation of nitrilase and nitrile hydratase biocatalytic systems, *Appl. Microbiol. Biotechnol.*, 64, 76, 2004.
43. Webster, N.A., Ramsden, D.K., and Hughes, J., Comparative characterisation of two *Rhodococcus* species as potential biocatalysts for ammonium acrylate production, *Biotechnol. Lett.*, 23, 95, 2001.
44. Okamoto, M. and Nagano, O., Jpn. Kokai Tokkyo Koho JP 2004305062 A2, 2004.
45. Symes, K.C. and Hughes, J., U.S. Patent 6,162,624, 2000.
46. Poltavskaya, S.V., Kozulina, T.N., Singirtsev, I.N., Kozulin, S.V., Shub, G.M., and Voronin, S.P., Development and implementation of biocatalytic method for acrylic acid manufacturing. I. Isolation of *Alcaligenes denitrificans* strain transforming acrylonitrile into ammonium acrylate. Optimization of the culture medium, *Biotechnologiya*, 1, 62, 2004.
47. Chauhan, S., DiCosimo, R., Fallon, R.D., Gavagan, J.E., and Payne, M.S., U.S. Patent 6416980 B1, 2002.
48. Chauhan, S., DiCosimo, R., Fallon, R., Gavagan, J., Manzer, L.E., and Payne, M.S., U.S. Patent 6,582,943 B1, 2003.
49. Dadd, M.R., Claridge, T.D.W., Pettman, A.J., and Knowles, C.J., Biotransformation of benzonitrile to benzohydroxamic acid by *Rhodococcus rhodochrous* in the presence of hydroxylamine, *Biotechnol. Lett.*, 23, 221, 2001.
50. Subramanian, V., Christenson, C.P., Conboy, C.B., Li, K., Redwine, O.D., Chopin, L.J., III, Miller, B.A., Morabito, P.L., Winterton, R.C., and Rosner, B.M., PCT Int. Appl. WO 2004097028 A2, 2004.
51. Osswald, S., Wajant, H., and Effenberger, F., Characterization and synthetic applications of recombinant AtNIT1 from *Arabidopsis thaliana*, *Eur. J. Biochem.*, 269, 680, 2002.
52. Effenberger, F. and Osswald, S., Enantioselective hydrolysis of (*RS*)-2-fluoroarylacetonitriles using nitrilase from *Arabidopsis thaliana*, *Tetrahedron Asymmetry*, 12, 279, 2001.
53. Yamada, H., Shimizu, S., and Kobayashi, M., Hydratases involved in nitrile conversion: screening, characterization and application, *Chem. Rec.*, 1, 152, 2001.
54. Martinkova, L. and Mylerova, V., Synthetic applications of nitrile-converting enzymes, *Curr. Org. Chem.*, 7, 1279, 2003.
55. Martinkova, L. and Kren, V., Nitrile- and amide-converting microbial enzymes: stereo-, regio- and chemoselectivity, *Biocatal. Biotransform.*, 20, 73, 2002.
56. Banerjee, A., Sharma, R., and Banerjee, U.C., The nitrile-degrading enzymes: current status and future prospects, *Appl. Microbiol. Biotechnol.*, 60, 33, 2002.
57. Huang, W., Jia, J., Cummings, J., Nelson, M., Schneider, G., and Lindqvist, Y., Crystal structure of nitrile hydratase reveals a novel center in a novel fold, *Structure*, 5, 691, 1997.

58. Cowan, D.A., Cameron, R.A., and Tsekos, T.L., Comparative biology of mesophilic and thermophilic nitrile hydratases, *Adv. Appl. Microbiol.*, 52, 123, 2003.
59. Endo, I. and Odaka, M., Studies on photoreactive enzyme — nitrile hydratase, *Prog. Biotechnol.*, 22, 159, 2002.
60. Endo, I., Nojiri, M., Tsujimura, M., Nakasako, M., Nagashima, S., Yohda, M., and Odaka, M., Fe-type nitrile hydratase, *J. Inorg. Biochem.*, 83, 247, 2001.
61. Radu, S.D., Nitrile hydration by the cobalt-containing nitrile hydratase. DFT investigation of the mechanism, *Revista de Chimie* (Bucharest, Romania), 56, 359, 2005.
62. Kobayashi, M. and Shimizu, S., Nitrile hydrolases, *Curr. Opin. Chem. Biol.*, 4, 95, 2000.
63. Tsujimura, M., Odaka, M., Nakayama, H., Dohmae, N., Koshino, H., Asami, T., Hoshino, M., Takio, K., Yoshida, S., Maeda, M., and Endo, I., A novel inhibitor for Fe-type nitrile hydratase: 2-cyano-2-propyl hydroperoxide. *J. Am. Chem. Soc.*, 125, 11532, 2003.
64. Nagashima, S., Nakasako, M., Dohmae, N., Tsujishima, M., Takio, K., Odaka, M., Yohda, M., Kamiya, N., and Endo, I., Novel nonheme iron center of nitrile hydratase with a claw setting of oxygen atoms, *Nat. Struct. Biol.*, 5, 347, 1998.
65. Miyanaga, A., Fushinobu, S., Ito, K., and Wakagi, T., Crystal structure of cobalt-containing nitrile hydratase, *Biochem. Biophys. Res. Commun.*, 288, 1169, 2001.
66. Miyanaga, A., Fushinobu, S., Ito, K., Shoun, H., and Wakagi, T., Mutational and structural analysis of cobalt-containing nitrile hydratase on substrate and metal binding, *Eur. J. Biochem.*, 271, 429, 2004.
67. Hourai, S., Miki, M., Takashima, Y., Mitsuda, S., and Yanagi, K., Crystal structure of nitrile hydratase from a thermophilic *Bacillus smithii*, *Biochem. Biophys. Res. Comm.*, 312, 340, 2003.
68. Komeda, H., Kobayashi, M., and Shimizu, S., Characterization of the gene cluster of high-molecular-mass nitrile hydratase (H-NHase) induced by its reaction product in *Rhodococcus rhodochrous* J1, *Proc. Natl. Acad. Sci. USA*, 93, 4267, 1996.
69. Stevens, J.M., Rao Saroja, N., Jaouen, M., Belghazi, M., Schmitter, J.-M., Mansuy, D., Artaud, I., and Sari, M.-A., Chaperone-assisted expression, purification, and characterization of recombinant nitrile hydratase NII from *Comamonas testosteroni*, *Protein Expression Purif.*, 29, 70, 2003.
70. Yokoyama, M., Kashiwagi, M., Iwasaki, M., Fuhshuku, K., Ohta, H., and Sugai, T., Realization of the synthesis of α,α -disubstituted carbamylacetates and cyanoacetates by either enzymatic or chemical functional group transformation, depending upon the substrate specificity of *Rhodococcus amidase*, *Tetrahedron Asymmetry*, 15, 2817, 2004.
71. Wu, Z.-L. and Li, Z.-Y., Practical synthesis of optically active α,α -disubstituted malonamic acids through asymmetric hydrolysis of malonamide derivatives with *Rhodococcus* sp. CGMCC 0497, *J. Org. Chem.*, 68, 2479, 2003.
72. Wang, M.-X., Feng, G.-Q., and Zheng, Q.-Y., Synthesis of high enantiomeric pure gem-dihalocyclopropane derivatives from biotransformations of nitriles and amides, *Tetrahedron Asymmetry*, 15, 347, 2004.
73. Wang, M.-X., Feng, G.-Q., and Zheng, Q.-Y., Nitrile and amide biotransformations for efficient synthesis of enantiopure gem-dihalocyclopropane derivatives, *Adv. Synth. Catal.*, 345, 695, 2003.
74. Wang, M.-X. and Feng, G.-Q., Nitrile biotransformation for highly enantioselective synthesis of 3-substituted 2,2-dimethylcyclopropanecarboxylic acids and amides, *J. Org. Chem.*, 68, 621, 2003.
75. Wang, M.-X. and Lin, S.-J., Practical and convenient enzymatic synthesis of enantiopure α -amino acids and amides, *J. Org. Chem.*, 67, 6542, 2002.
76. Wang, M.-X., Lin, S.-J., Liu, J., and Zheng, Q.-Y., Efficient biocatalytic synthesis of highly enantiopure α -alkylated arylglycines and amides, *Adv. Synth. Catal.*, 346, 439, 2004.
77. Wang, M.-X., Li, J.-J., Ji, G.-J., and Li, J.-S., Enantioselective biotransformations of racemic 2-aryl-3-methylbutyronitriles using *Rhodococcus* sp. AJ270, *J. Mol. Catal. B Enzym.*, 14, 77, 2001.
78. Hensel, M., Lutz-Wahl, S., and Fischer, L., Stereoselective hydration of (RS)-phenylglycine nitrile by new whole cell biocatalysts, *Tetrahedron Asymmetry*, 13, 2629, 2002.
79. Ramer, S.W., Huisman, G., Millis, J., Sheldon, R., Del Cardayre, S., Tobin, M., Cox, A., and Davis, S.C., U.S. Patent Appl. 20020137153 A1, 2002.

80. Ishikawa, T. and Hayakawa, K., Jpn. Kokai Tokkyo Koho JP 2004254690 A2, 2004.
81. Nagasawa, T. and Matsuyama, A., Jpn. Kokai Tokkyo Koho JP 2004081169 A2, 2004.
82. Osprian, I., Fechter, M.H., and Griengl, H., Biocatalytic hydrolysis of cyanohydrins: an efficient approach to enantiopure α -hydroxy carboxylic acids, *J. Mol. Catal. B Enzym.*, 24–25, 89, 2003.
83. Griengl, H., Osprian, I., Schoemaker, H.E., Reisinger, C., and Schwab, H., PCT Int. Appl. WO 2004076385 A2, 2004.
84. Poechlauer, P., Skranc, W., and Wubbolts, M., The large-scale biocatalytic synthesis of enantio-pure cyanohydrins, in *Asymmetric Catalysis on Industrial Scale*, Blaser, H.-U. and Schmidt, E., Eds., Wiley-VCH, Weinheim, Germany, 2004, p. 151.
85. Schwab, H., Glieder, A., Kratky, C., Dreveny, I., Poechlauer, P., Skranc, W., Mayrhofer, H., Wirth, I., Neuhofer, R., and Bona, R., U.S. Patent 6,861,243 B2, 2005.
86. Reisinger, C., Osprian, I., Glieder, A., Schoemaker, H.E., Griengl, H., and Schwab, H., Enzymatic hydrolysis of cyanohydrins with recombinant nitrile hydratase and amidase from *Rhodococcus erythropolis*, *Biotechnol. Lett.*, 26, 1675, 2004.
87. Preiml, M., Hillmayer, K., and Klempier, N., A new approach to β -amino acids: biotransformation of *N*-protected β -amino nitriles, *Tetrahedron Lett.*, 44, 5057, 2003.
88. Preiml, M., Hönig, H., and Klempier, N., Biotransformation of β -amino nitriles: the role of the *N*-protecting group, *J. Mol. Catal. B Enzym.*, 29, 115, 2004.
89. Winkler, M., Martinkova, L., Knall, A.C., Krahulec, S., and Klempier, N., Synthesis and microbial transformation of β -amino nitriles, *Tetrahedron*, 61, 4249, 2005.
90. Hann, E.C., Sigmund, A.E., Fager, S.K., Cooling, F.B., Gavagan, J.E., Ben-Bassat, A., Chauhan, S., Payne, M.S., Hennessey, S.M., and DiCosimo, R., Biocatalytic hydrolysis of 3-hydroxyalkanenitriles to 3-hydroxyalkanoic acids, *Adv. Synth. Catal.*, 345, 775, 2003.
91. DiCosimo, R., Fallon, R., Gavagan, J.E., and Manzer, L., U.S. Patent Appl. 2003148480 A1, 2003.
92. Chauhan, S., DiCosimo, R., Fallon, R., Gavagan, J., Manzer, L.E., and Payne, M.S., U.S. Patent 6,582,943 B1, 2003.
93. Chassin, C., A biotechnological process for the production of nicotinamide, *Chim. Oggi*, 14, 9, 1996.
94. Shaw, N.M., Robins, K.T., and Kiener, A., Lonza: 20 years of biotransformations, *Adv. Synth. Catal.*, 345, 425, 2003.
95. Heveling, J., Armbruster, E., Utiger, L., Rohner, M., Dettwiler, H.-R., and Chuck, R.J., U.S. Patent 5,719,045, 1998.
96. Anonymous *Chem. Eng. News*, 81(23), 12, 2003.
97. Robins, K.T. and Nagasawa, T., U.S. Patent 6,444,451 B1, 2002.
98. Robins, K.T. and Nagasawa, T., U.S. Patent Appl. 20030148478 A1, 2003.
99. Robins, K.T. and Nagasawa, T., U.S. Patent Appl. 20040142447 A1, 2004.
100. Shapiro, R., DiCosimo, R., Hennessey, S.M., Stieglitz, B., Campopiano, O., and Chiang, G.C., Discovery and development of a manufacturing-scale synthesis of azafenidin, *Org. Process Res. Dev.*, 5, 593, 2001.
101. Bernet, N., Arnaud, A., and Galzy, P., Optimization of culture conditions of *Brevibacterium* sp. A4 for the production of nitrile hydratase, *Biocatalysis*, 3, 259, 1990.
102. Ingvorsen, K., Godtfredsen, S.E., and Tsuchiya, R.T., Microbial hydrolysis of organic nitriles and amides, *Ciba Found. Symp.*, 140, 16, 1988.
103. Andresen, O. and Godtfredsen, S. E., European Patent EP 178106 B1, 1993.
104. Nagamune, T., Kurata, H., Hirata, M., Honda, J., Hirata, A., and Endo, I., Photosensitive phenomena of nitrile hydratase of *Rhodococcus* sp. N-771, *Photochem. Photobiol.*, 51, 87, 1990.
105. Honda, J., Kandori, H., Okada, T., Nagamune, T., Shichida, Y., Sasabe, H., and Endo, I., Spectroscopic observation of the intramolecular electron transfer in the photoactivation processes of nitrile hydratase, *Biochemistry*, 33, 3577, 1994.
106. Nagasawa, T., Nanba, H., Ryuno, K., Takeuchi, K., and Yamada, H., Nitrile hydratase of *Pseudomonas chlororaphis* B23: purification and characterization, *Eur. J. Biochem.*, 162, 691, 1987.
107. Yamada, H., Ryuno, K., Nagasawa, T., Enomoto, K., and Watanabe, I., Optimum culture conditions for production by *Pseudomonas chlororaphis* B23 of nitrile hydratase, *Agric. Biol. Chem.*, 50, 2859, 1986.

108. Asano, Y., Yasuda, T., Tani, Y., and Yamada, H., Microbial degradation of nitrile compounds. Part VII. A new enzymic method of acrylamide production, *Agric. Biol. Chem.*, 46, 1183, 1982.
109. Ashina, Y. and Suto, M., Development of an enzymic process for manufacturing acrylamide and recent progress, *Bioprocess Technol.*, 16, 91, 1993.
110. Kobayashi, M., Nagasawa, T., and Yamada, H., Enzymic synthesis of acrylamide: a success story not yet over, *Trends Biotechnol.*, 10, 402, 1992.
111. Nagasawa, T., Ryuno, K., and Yamada, H., Superiority of *Pseudomonas chlororaphis* B23 nitrile hydratase as a catalyst for the enzymic production of acrylamide, *Experientia*, 45, 1066, 1989.
112. Ryuno, K., Nagasawa, T., and Yamada, H., Isolation of advantageous mutants of *Pseudomonas chlororaphis* B23 for the enzymic production of acrylamide, *Agric. Biol. Chem.*, 52, 1813, 1988.
113. DiCosimo, R., Hann, E.C., Eisenberg, A., Fager, S.K., Perkins, N.E., Gallagher, F.G., Cooper, S.M., Gavagan, J.E., Stieglitz, B., and Hennessey, S.M., Biocatalytic production of 5-cyanovaleramide from adiponitrile, *ACS Symp. Ser.*, 767, 114, 2000.
114. Hann, E.C., Eisenberg, A., Fager, S.K., Perkins, N.E., Gallagher, F.G., Cooper, S.M., Gavagan, J.E., Stieglitz, B., Hennessey, S.M., and DiCosimo, R., 5-Cyanovaleramide production using immobilized *Pseudomonas chlororaphis* B23, *Bioorg. Med. Chem.*, 7, 2239, 1999.
115. Watanabe, F., Jpn. Kokai Tokkyo Koho JP 2004215513 A2, 2004.
116. Watanabe, F., Jpn. Kokai Tokkyo Koho JP 2004222538 A2, 2004.
117. Ramey, S. and Tichenor, G., Enzymatically produced acrylamide: improving the environment, an additional benefit of building a better product, WEFTEC.02 Conference Proceedings, Chicago, September 28, 2002.
118. Wieser, M. and Pommares, P., PCT Int. Appl. WO 2003066800 A3, 2004.
119. Furuya, K., Tamaki, A., Nagasawa, S., and Suzuki, A., PCT Int. Appl. WO 2004108942 A1, 2004.
120. Yamaki, T., Banba, S., Matoishi, K., Ito, K., Kobayashi, H., Tanaka, E., and Oikawa, T., PCT Int. Appl. WO 2004056990 A1, 2004.
121. Petrillo, K.L., Wu, S., Hann, E.C., Cooling, F.B., Ben-Bassat, A., Gavagan, J.E., DiCosimo, R., and Payne, M.S., Over-expression in *Escherichia coli* of a thermostable nitrile hydratase from *Comamonas testosteroni* 5-MGAM-4D, *App. Microbiol. Biotechnol.*, 67, 664, 2005.
122. Mersinger, L.J., Hann, E.C., Cooling, F.B., Ben-Bassat, A., Gavagan, J.E., Wu, S., Petrillo, K.L., Payne, M.S., and DiCosimo, R., Production of acrylamide using alginate-immobilized *E. coli* expressing *Comamonas testosteroni* 5-MGAM-4D nitrile hydratase, *Adv. Synth. Catal.*, 347, 1125, 2005.
123. Nagasawa, T., Shimizu, H., and Yamada, H., The superiority of the third-generation catalyst, *Rhodococcus rhodochrous* J1 nitrile hydratase, for industrial production of acrylamide, *Appl. Microbiol. Biotechnol.*, 40, 189, 1993.
124. Padmakumar, R. and Oriel, P., Bioconversion of acrylonitrile to acrylamide using a thermostable nitrile hydratase, *Appl. Biochem. Biotechnol.*, 77–79, 671, 1999.
125. Graham, D., Pereira, R., Barfield, D., and Cowan, D., Nitrile biotransformations using free and immobilized cells of a thermophilic *Bacillus* spp., *Enz. Microb. Technol.*, 26, 368, 2000.
126. Han, J., Zhang, Y., Xue, J., Huang, J., and Chen, X., Research on manufacture of acrylamide on industrial scale by microbial method, *Shanghai Huagong*, 20, 7, 1995.
127. Liu, M., Li, C., Gao, Y., Wang, Y., and Cao, Z., Optimization of glucose- Co^{2+} coupling fed batch fermentation for production of nitrile hydratase of high activity by *Nocardia* sp. RS, *Guocheng Gongcheng Xuebao*, 3, 555, 2003.
128. Liu, M., Li, C., Gao, Y., Huang, Y., and Cao, Z., Improved process for high acrylamide accumulation with acclimated microorganism *Nocardia* sp., *Huagong Xuebao*, 55, 1678, 2004.
129. Liu, M., Li, C., Huang, Y., Gao, Y., and Cao, Z., Improving the acrylamide-tolerance of nitrile hydratase in *Nocardia* sp. by extreme cultivation, *Guocheng Gongcheng Xuebao*, 4, 250, 2004.
130. Shi, Y., Yu, H., Sun, X., Tian, Z., and Shen, Z., Cloning of the nitrile hydratase gene from *Nocardia* sp. in *Escherichia coli* and *Pichia pastoris* and its functional expression using site-directed mutagenesis, *Enzyme Microb. Technol.*, 35, 557, 2004.
131. Sun, X., Shi, Y., Yu, H., and Shen, Z., Bioconversion of acrylonitrile to acrylamide using hollow-fiber membrane bioreactor system, *Biochem. Eng. J.*, 18, 239, 2004.
132. Murao, K. and Ishii, K., U.S. Patent Appl. 2004048348 A1, 2004.

133. Kim, B.-Y. and Hyun, H.-H., Production of acrylamide using immobilized cells of *Rhodococcus rhodochrous* M33. *Biotechnol. Bioprocess Eng.*, 7, 194, 2002.
134. Tauber, M.M., Cavaco-Paulo, A., Robra, K.H., and Gubitz, G.M., Nitrile hydratase and amidase from *Rhodococcus rhodochrous* hydrolyze acrylic fibers and granular polyacrylonitriles, *Appl. Environ. Microbiol.*, 66, 1634, 2000.
135. Wang, N., Xu, Y., Lu, D.-N., and Xu, J.-H., Enzymatic surface modification of acrylic fiber, *AATCC Review*, 4, 28, 2004.
136. Battistel, E., Morra, M., and Marinetti, M., Enzymatic surface modification of acrylonitrile fibers, *Appl. Surf. Sci.*, 177, 32, 2001.
137. Snajdrova, R., Kristova-Mylerova, V., Crestia, D., Nikolaou, K., Kuzma, M., Lemaire, M., Gallienne, E., Bolte, J., Bezouska, K., Kren, V., and Martinkova, L., Nitrile biotransformation by *Aspergillus niger*, *J. Mol. Catal. B Enzym.*, 29, 227, 2004.

2 Biocatalytic Deracemization: Dynamic Resolution, Stereoinversion, Enantioconvergent Processes, and Cyclic Deracemization

Yolanda Simeó, Wolfgang Kroutil, and Kurt Faber

CONTENTS

2.1	Introduction	27
2.2	Improving Kinetic Resolution	28
2.2.1	Re-Racemization and Repeated Resolution.....	28
2.2.2	Dynamic Resolution.....	32
2.3	Stereoinversion.....	36
2.4	Enantioconvergent Processes	37
2.4.1	Enantioconvergence Using Two Biocatalysts.....	40
2.4.2	Enantioconvergence Using Combined Bio- and Chemocatalysis.....	42
2.4.3	Enantioconvergence Using a Single Biocatalyst.....	43
2.5	Deracemization by a Cyclic Oxidation–Reduction Sequence	45
2.6	Summary.....	46
	Acknowledgment.....	47
	References	47

2.1 INTRODUCTION

Driven by the increased demand for chiral drugs in enantiomerically pure form, following the release of Food and Drug Administration's (FDA) marketing guidelines, the search for novel catalytic methods to access enantiopure compounds is a major topic in contemporary organic synthesis [1–3]. In this context, biocatalysis has been applied in preparative organic chemistry at an increasing pace over the last two decades [4–7]. From the two principles of biocatalytic reactions where chiral substrates are involved—(i) desymmetrization of *meso*- and prochiral compounds [8,9]; and (ii) kinetic resolution of racemates [10–13]—the latter is remarkably dominant in number of applications (~1:4) [14]. This is because (out of combinatorial considerations) there are more racemic molecules possible than *meso*- and prochiral compounds, which bear an element of symmetry within the molecule. Consequently, racemates always have been (and will be) an indispensable starting point

for the synthesis of chiral materials in nonracemic form. In principle, kinetic resolution of racemates is based on the difference in reaction rates of enantiomers with a chiral (bio)catalyst. In an ideal case, the reactive “fitting” enantiomer is quickly converted, while the “wrong” enantiomer remains untouched. Thus, the reaction comes to a standstill at 50% conversion, where both enantiomeric substrate and product can be separated by physical means.

Despite its widespread application, kinetic resolution is impeded by several inherent disadvantages, especially on an industrial scale. The most obvious drawbacks of kinetic resolution are:

1. The theoretical yield of each enantiomer can never exceed a limit of 50%.
2. Separation of the formed product from the remaining substrate may be laborious in particular for cases in which simple extraction or distillation fails and chromatographic methods are required [15].
3. In the majority of processes, only one stereoisomer is desired and there is little or no use for the other. In some rare cases, the unwanted isomer may be used through a separate synthetic pathway in an enantioconvergent fashion, but this requires a highly flexible synthetic strategy [16].
4. For kinetic reasons, the optical purity of the substrate and the product is depleted at the point, where separation of product and substrate is most desirable from a preparative point of view, i.e., at 50% conversion [17].

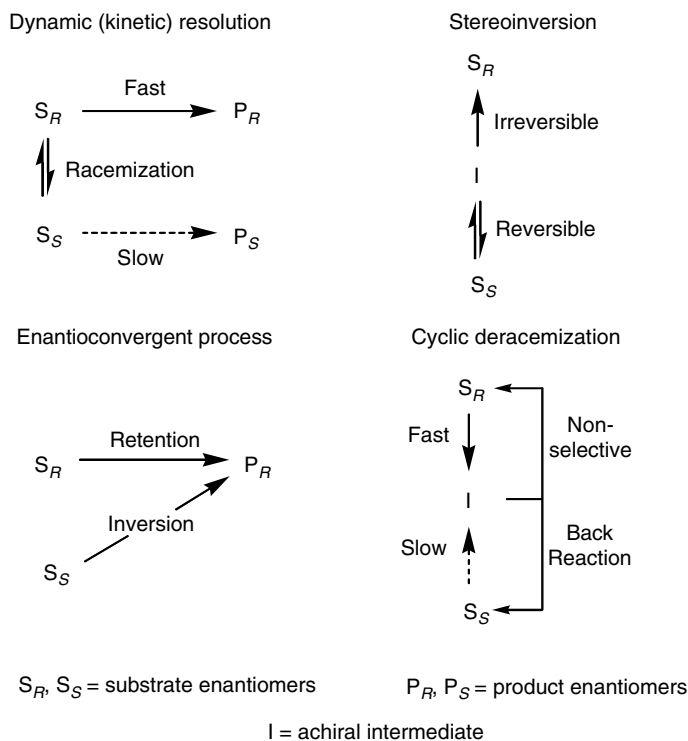
After all, it should be kept in mind that an ideal production process leads to a single enantiomeric product in 100% yield. So alternatives to kinetic resolution techniques that can provide a single stereoisomer from a racemate are highly advantageous as they provide a double theoretical yield of the desired stereoisomeric product [18]. These latter processes are encompassed under the general term “deracemization” [19–21] (Scheme 2.1). All of these techniques deal with a common stereochemical phenomenon, i.e., both substrate enantiomers have to be processed through two different pathways. In this chapter, general strategies that lead to the formation of a single enantiomeric product in 100% theoretical yield from a racemate are reviewed.

2.2 IMPROVING KINETIC RESOLUTION

2.2.1 RE-RACEMIZATION AND REPEATED RESOLUTION

To avoid the loss of half of the material in kinetic resolution, it has been a common practice to racemize the unwanted stereoisomer after separation from the desired product and to subject it again to kinetic resolution in a subsequent cycle until virtually all of the racemic material has been converted into a single stereoisomer [22,23]. On a superficial look, repeated resolution appears to be less than optimal and certainly lacks synthetic elegance, bearing in mind that an infinite number of cycles are theoretically required to transform all of the racemic starting material into a single stereoisomer. However, it is a viable option for resolutions on an industrial scale, in particular for continuous processes, where the racemized material is simply fed back into the subsequent batch of the resolution process.

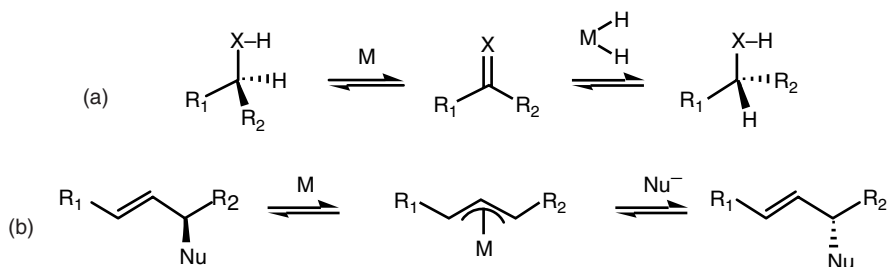
Racemization, in general, is an energetic “downhill” reaction due to an increase of entropy [24], and thus has been considered more often as an undesired side reaction rather than as a synthetically useful transformation. As a consequence, the controlled racemization of organic compounds has been scarcely studied deliberately, and a significant part of the data available to date stems from industrial research predominantly reported in the patent literature. It was only recently that the importance of synthetic



SCHEME 2.1 General strategies for the transformation of a racemate into a single stereoisomeric product.

protocols for the controlled racemization of organic compounds has been recognized, as highlighted by the review of Zwanenburg et al. [25]. A detailed investigation of the data available reveals that chemical racemization techniques largely depend on harsh reaction conditions, predominantly (i) thermal racemization, as well as (ii) strong acid catalysis or (iii) base catalysis [26], and (iv) through chirally labile intermediates [25]. Overall, ~75% of all racemizations fall under these categories. As a consequence of the harsh reaction conditions, the possibility for process control in chemical racemization is very limited and undesired side reactions, such as elimination, condensation, rearrangement, and/or decomposition, set a low ceiling on the preparative utility of these processes. However, milder methods for chemical racemization have been recently developed [27]. For instance, chiral *sec*-alcohols and amines can be racemized through a transition metal-catalyzed oxidation–reduction sequence [28] or by π -allyl formation [29] (Scheme 2.2). The mechanism of transition metal-catalyzed racemization by hydrogen transfer has been extensively investigated [30], and a recent study indicates that two different hydridic pathways can be involved in these reactions [31]: a metal monohydride mechanism and a metal dihydride mechanism. Whereas the first mechanism applies to rhodium, iridium, and most nonhalide ruthenium complexes, the second mechanism operates for ruthenium dihalide catalyst precursors.

An example of a ruthenium catalyst is shown in Scheme 2.3, which is one of the few Ru-complexes that are applicable to a broad substrate spectrum. A relevant feature of this complex is that no external base is needed as a co-catalyst, since one of the oxygens of



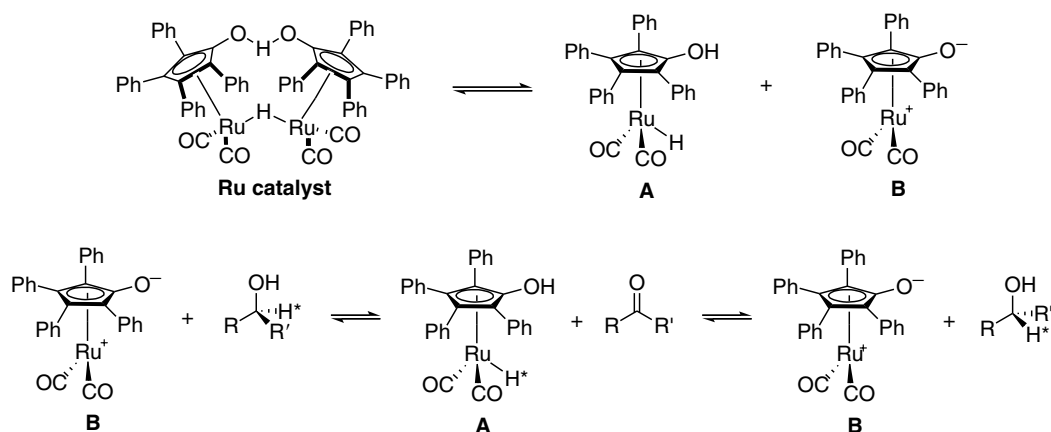
SCHEME 2.2 (a) Racemization of alcohols (X = O) and amines (X = NH) by transition metal-catalyzed hydrogen transfer (M = Rh, Ir, Ru). (b) Racemization of allylic alcohols by π -allyl mechanism (M = Ru, Pd; Nu = acetate).

the ligand acts as a base. Thus, the reaction of the basic oxygen with an alcohol gives rise to ruthenium hydride intermediate “A” and the ketone, which is reduced by ruthenium intermediate “A” to form the racemic alcohol and ruthenium species “B.” A similar mechanism with the formation of ruthenium amine intermediates has been recently proposed for the dehydrogenation of amines to imines [32,33].

However, most hydrogen transfer catalysts need the addition of an external base as co-catalyst, which can affect the performance of the enzyme and can also cause side reactions on substrates and/or products. For instance, the presence of base in the resolution of β -haloalcohols can generate epoxides. As a consequence, it is advisable to use a catalyst that acts without external base.

On the other hand, racemization of allylic esters has been accomplished with (π -allyl)palladium complexes using Pd(0) catalysis. This isomerization can proceed through different mechanisms [34]. Interconversion of enantiomers of *sec*-amines is also performed by Pd/C-catalyzed dehydrogenation–hydrogenation (Scheme 2.7c).

Another strategy for racemization is to (chemically) convert compounds bearing a stable stereocenter into a chirally unstable intermediate. For instance, amino acid amides or -esters can be racemized via Schiff-base derivatives of aromatic aldehydes involving the α -amino group [35] or, alternatively, for free amino acids, mixed acid anhydrides are used. In this sense, several racemic unsaturated amino acids have been resolved by hydrolysis of



SCHEME 2.3 Racemization of alcohols catalyzed by an Ru catalyst.

the corresponding amino acid amide racemic mixture with an aminopeptidase from *Pseudomonas putida* ATCC 12633 and subsequent Schiff-base formation of the remaining amide enantiomer [36]. However, after acid/base-catalyzed racemization of the respective intermediates, the starting compound has to be liberated again, which overall turns this technique into a rather tedious multistep procedure.

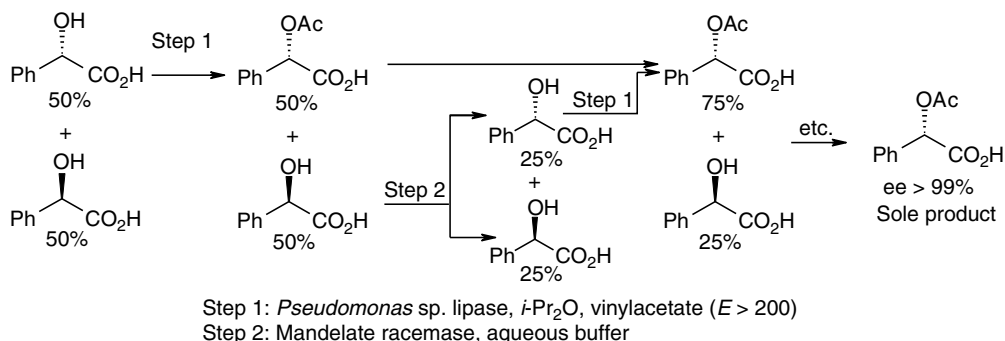
The general disadvantage of most of these techniques based on chemocatalysis—i.e., the requirement of harsh reaction conditions—can be avoided by the employment of biocatalytic racemization methods, which proceed under mild reaction conditions, e.g., room temperature, atmospheric pressure, and physiological pH. Under these conditions, side reactions are largely suppressed [37]. Unfortunately, Nature does not rely on racemates to a large extent and, as a consequence, biochemical racemization is a rather scarce feature, making racemases a small group of enzymes that can only be found in certain biological niches. One of the major targets for biochemical racemization involves stereogenic centers in carbohydrates, i.e., *sec*-alcohol groups. However, since both stereoisomers of these reactions represent diastereomers rather than enantiomers, “epimerization” would be a more correct term. Various enzymes—epimerases—are involved in the racemization of *sec*-hydroxyl groups. These enzymes are usually NAD⁺-dependent and of very little interest for practical applications.

Among noncarbohydrate “true” racemases, base catalysis seems to be the general scheme of biochemical racemization, and two major groups can be classified according to their reaction mechanism [38]: (i) racemases employing a one-base mechanism, i.e., the proton at the chiral center is abstracted by the same base functionality of the enzyme, which re-adds it—and (ii) those employing a two-base mechanism, i.e., one base is capable of *abstracting* the proton at the chiral center and another base *puts it back* from the opposite side, thus resembling a ping-pong mechanism.

Mandelate racemase [EC 5.1.2.2] belongs to the latter group employing a two-base mechanism [39]. The Mg²⁺-dependent enzyme is capable of racemizing a remarkably broad substrate spectrum, which opens up large possibilities for the deracemization of various kinds of α -hydroxyacids. Substrates that meet the following constraints are accepted by mandelate racemase [40]:

1. The α -hydroxyacid moiety is (almost strictly) required, as the only exception to this rule seems to be an α -hydroxy carboxamide functionality [41].
2. A π -electron system has to be present in the β,γ -position. The latter can be freely varied, including the corresponding α -hydroxy- β,γ -alkenoic and α -hydroxy- α -aryl carboxylic acids. Even heteroaromatic systems are accepted at reasonable rates [42]. In general, electron-withdrawing groups attached to the β,γ -unsaturated (or aromatic) system that help to stabilize the (anionic) transition intermediate through resonance, increase the reaction rates significantly [43].

An example of mandelate racemase application, based on a two-enzyme system consisting of (i) a lipase-catalyzed enantioselective acylation followed by (ii) mandelate racemase-catalyzed racemization of the remaining nonreacted substrate enantiomer, is shown in [Scheme 2.4](#) [44]. Thus, in the first step, (\pm)-mandelic acid is subjected to lipase-catalyzed *O*-acylation in an organic solvent producing (*S*)-*O*-acetyl mandelate, leaving the (*R*)-enantiomer behind. Due to the high selectivity ($E > 200$), the reaction comes to a standstill at 50% conversion. In the second step, the organic solvent is switched to aqueous buffer [45], and the remaining unreacted (*R*)-mandelic acid is racemized in the presence of (*S*)-*O*-acetyl mandelate, which is a nonsubstrate. When this two-step process is repeated four times, (*S*)-*O*-acetyl mandelate is obtained in ~80% chemical yield and >99% enantiomeric



SCHEME 2.4 Deracemization of mandelate by repeated resolution, employing a lipase–racemase two-enzyme process.

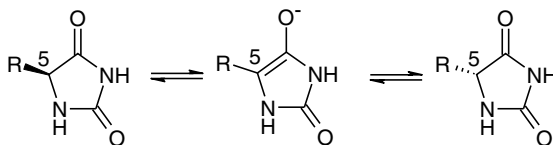
excess as the sole product. It should be emphasized that separation of the formed product from the remaining starting material is not required due to the high specificity of the racemase employed.

A relevant group of racemases are amino acid racemases, which are widely used in industry because of the high commercial importance of amino acids and their derivatives. Although the biosynthesis of α -amino acids is highly stereospecific with respect to the L-stereoisomers, an impressive number of D-analogs have been found in various biological sources, usually as components of highly potent natural products [46,47]. Instead of a *de novo* biosynthesis through “mirror-image” metabolic pathways, D-amino acid isomers are generally obtained by biocatalytic racemization and kinetic resolution; thus it is not surprising that racemases acting on α -amino acids and derivatives thereof are quite common.

Another remarkable group of isomerase enzymes are hydantoin racemases. Monosubstituted hydantoins bearing an aromatic substituent in position 5, e.g., phenylhydantoin (Scheme 2.5, R = Ph), spontaneously racemize under weak alkaline conditions [48]. It has been discussed that the velocity of chemical racemization is influenced by the electronic nature of the substituent, such as its electronegativity and the presence of adjacent π -systems, which facilitates enolate stabilization due to participation of mesomeric structures [49,50]. As a consequence, 5-alkyl- or 5-aryl-alkyl-substituted hydantoins are chirally stable, and spontaneous racemization is too slow to allow dynamic kinetic resolution. For these types of substrates, hydantoin racemases were found in *Arthrobacter* and *Pseudomonas* species, which enzymatically catalyze the interconversion of enantiomers. Hydantoin racemases find their application in the so-called hydantoinase process for the industrial-scale production of either D- (or L-) amino acids, depending on the follow-up “hydantoinase enzymes” used (the recommended name of these enzymes is “dihydropyrimidinase” [EC 3.5.2.2]).

2.2.2 DYNAMIC RESOLUTION

The disadvantages of kinetic resolution can largely be avoided by employing a so-called dynamic resolution [51–53] (Scheme 2.6). Such a process comprises kinetic resolution with an additional feature—*in situ* racemization of the starting material—which is usually achieved using chemocatalysis. Ultimately, both the substrate enantiomers $S_R + S_S$ are transformed into a single product enantiomer P_R in 100% theoretical yield. In contrast to kinetic resolution, where the reaction slows down at 50% conversion (or comes even to a standstill, if the enantioselectivity is sufficiently high), when the fast-reacting enantiomer S_R is

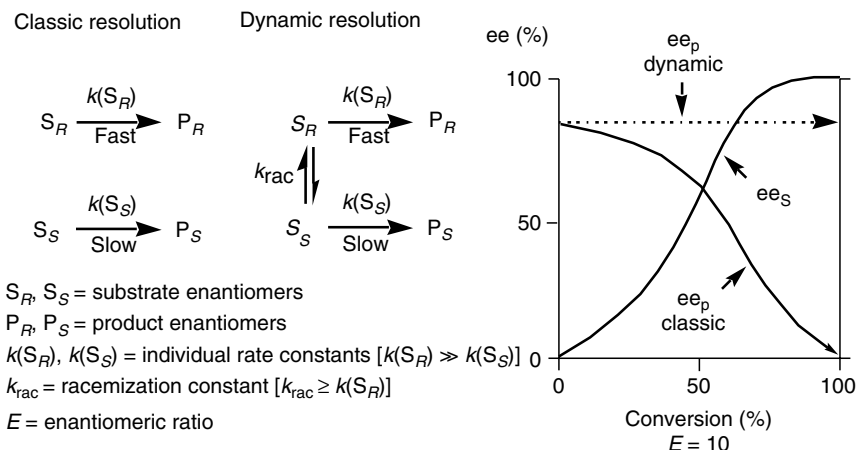


SCHEME 2.5 Chemical racemization of 5-substituted hydantoins via enolates.

consumed and only the slow-reacting counterpart S_S remains, substrate racemization ensures the continuous formation of S_R from S_S during the course of the reaction, and thus avoids the depletion of S_R . Therefore, the reaction does not come to a standstill and it can be run to completion by gradually converting all the racemic starting material into product P_R . In order to indicate the nonstatic behavior of such a process, the term “dynamic resolution” has been aptly coined. The major advantages of this methodology are twofold. The ee of the product is higher compared to classic resolutions and the separation of the substrate–product mixture can be omitted, due to the absence of an unwanted remaining substrate enantiomer. This concept has been applied to a variety of compounds such as α -amino acids, hemithioacetal esters, α -(hetero)arylcarboxylic acid esters, α -substituted nitriles, cyanohydrins, α -substituted thioesters, as well as 4-substituted oxazolin-5-ones and thiazolin-5-ones.

The following properties are typical for dynamic resolution processes [54,55]. From Scheme 2.6 it can be seen that (for kinetic resolutions) the ee of the product (ee_P) is at its maximum at the onset of the reaction and gradually begins to decline as the slower reacting enantiomer is accumulated in the reaction mixture, in particular around halfway through the reaction. This depletion does not occur if the substrate is constantly racemized during the resolution process and, thus, in a dynamic resolution the ee_P is *not* a function of the conversion but remains constant throughout the reaction. Since the catalyst always faces a racemic starting material ($[S_R]$ always equals $[S_S]$), it is understandable that the selection of the faster-reacting enantiomer from the substrate remains a simple task, as opposed to kinetic resolution where depletion of S_R occurs.

In order to design a successful dynamic resolution process, both parallel reactions—kinetic resolution [$k(S_R) \gg k(S_S)$] and *in situ* racemization (k_{rac})—have to be carefully tuned, taking into account the following aspects:



SCHEME 2.6 Kinetic principles of kinetic and dynamic resolution.

1. The kinetic resolution should be irreversible in order to ensure high enantioselectivity.
2. The enantiomeric ratio [*E*-value, $E = k(S_R)/k(S_S)$] should be at least greater than 20. For biocatalyzed reactions, the “binding” of the substrate enantiomers (which can be largely neglected with chemical catalysts) usually plays an important role in the chiral selection process, and *E*-values of enzyme-catalyzed reactions are therefore defined through Michaelis–Menten kinetics: $E = (k_{\text{cat}}/K_m)_{S_R}/(k_{\text{cat}}/K_m)_{S_S}$.
3. To avoid depletion of S_R , racemization (k_{rac}) should be at least equal to, or greater than, the reaction rate of the fast enantiomer $k(S_R)$.
4. In case the selectivities are only moderate, k_{rac} should be greater than $k(S_R)$ by a factor of about 10.
5. For obvious reasons, any spontaneous side reactions involving the substrate enantiomers as well as racemization of the product should be absent.
6. Dynamic resolution is generally limited to compounds possessing one stereocenter. However, under certain circumstances, multicenter compounds can be processed as long as the stereocenters are (stereo)chemically very similar. In such a case, the reaction proceeds through several diastereomeric intermediates.

A common scenario for dynamic resolution processes based on enantioselective biocatalysis makes use of a combination of an enzyme-catalyzed kinetic resolution coupled to *in situ* racemization of the remaining substrate enantiomer through chemocatalysis. Four practical situations can be encountered:

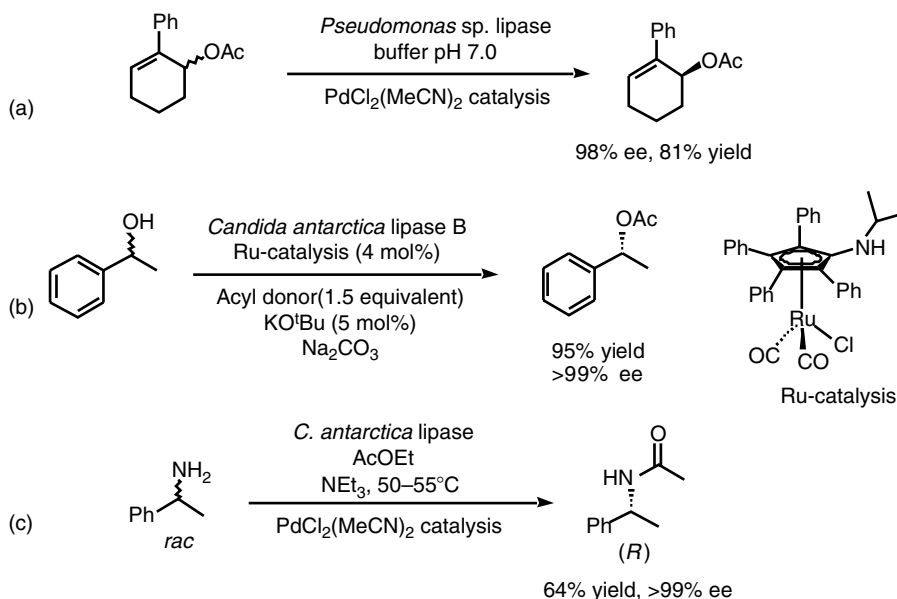
1. Compounds with a chirality center bearing an acidic proton—adjacent to an activating carbonyl group, such as an ester or ketone—usually undergo facile racemization through the formation of an achiral enolate species through base-catalyzed proton abstraction [56,57].
2. When such racemization is impossible, e.g., in case of a secondary alcohol, racemization can be achieved by a (reversible) decomposition reaction, such as the cleavage of hemi(thio)acetals and cyanohydrins [58,59].

If chemocatalysis relies on acid or base, its combination with the biocatalyst may be very difficult due to the incompatibility of enzymes with strong basic or acidic media. Consequently, biocompatible *in situ* racemization techniques are of high value. Typical examples include:

3. Transition metal-catalyzed racemization of *sec*-alcohols, based on Pd^{II} -catalyzed allylic rearrangement of allylic acetate esters [60] (Scheme 2.7a) or Ru-catalyzed oxidation–reduction sequences [61] (see Scheme 2.7b).
4. Benzylic amines can be racemized under biocompatible conditions using a reversible Pd/C-catalyzed dehydrogenation–hydrogenation reaction (see Scheme 2.7c) [62].

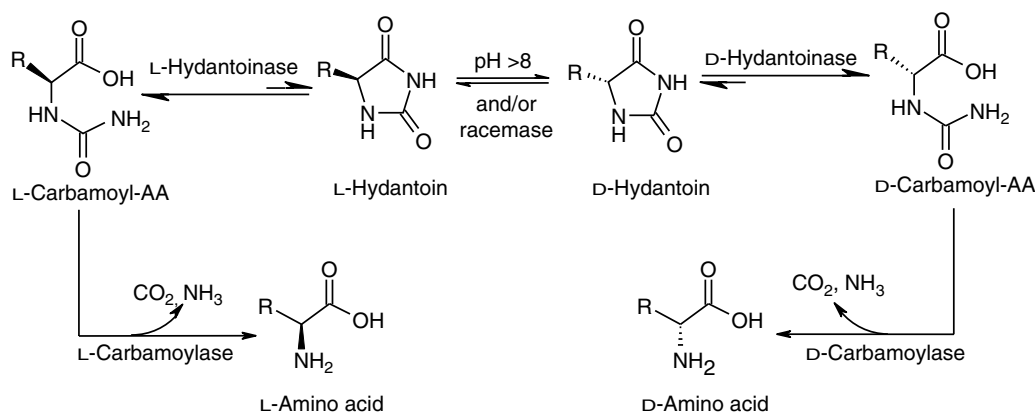
All of these techniques have been successfully applied to dynamic resolution. Some prominent examples are depicted in Scheme 2.7.

One approach to circumvent the incompatibility of chemical and biocatalysts for dynamic resolution consists in the combination of two biocatalysts, bearing in mind that enzymes are easily compatible with each other as they generally work under the same (physiological) reaction conditions. In this viewpoint, the application of racemases seems to be very promising.



SCHEME 2.7 (a) Dynamic resolution of an allylic *sec*-alcohol through combined Pd^{II}- and lipase catalysis. (b) Dynamic resolution of *sec*-alcohols through combined Ru- and lipase catalysis. (c) Dynamic resolution of a benzylic amine through Pd/C- and lipase catalysis.

One example of this strategy is the dynamic kinetic resolution of racemic hydantoins to either the D- (or L-) amino acid. This process consists of three steps (Scheme 2.8): (i) ring-opening hydrolysis of the hydantoin is performed by a D- (or L-) specific hydantoinase; (ii) carbamoylase-catalyzed hydrolysis of the resulting *N*-carbamoylamino acid; which shifts the equilibrium towards (iii) completion. For complete transformation of the *rac*-starting material in a dynamic kinetic resolution, substrate racemization is performed by chemical and/or enzymatic catalysis using a hydantoin racemase. As three enzymes are involved in this process, the relative activity (or expression level) of the enzymes must match the kinetic



SCHEME 2.8 Hydantoinase process for production of D- (or L-) amino acids by dynamic kinetic resolution.

criteria [63], i.e., (i) racemization of the hydantoin must be fast enough to meet (or exceed) the rate of hydantoin hydrolysis; and (ii) the degradation of the carbamoyl amino acid must be equal to, or higher than, its formation to avoid its accumulation, which would cause reformation of the hydantoin.

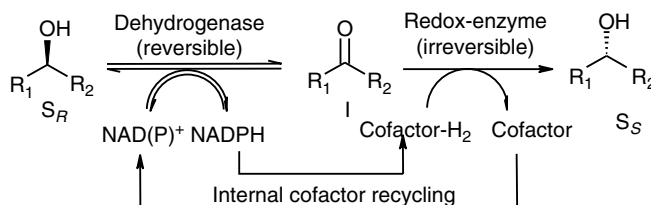
The classic hydantoinase process was first introduced in the 1970s for the production of D-amino acids such as D-phenylglycine and D-*p*-OH-phenylglycine [64]. Today, it is commercially applied at a scale of >1000 t/y for the above-mentioned amino acids, which are used as side chains for β -lactam antibiotics such as ampicillin and amoxicillin, respectively. It was only recently that the hydantoin process also became feasible on a large scale for L-amino acids due to the improvement of productivity by using a recombinant whole-cell biocatalyst [65], which allows commercialization even for low-priced amino acids such as L-methionine.

2.3 STEREOINVERSION

The difficulty to achieve *in situ* racemization with compounds possessing a configurationally stable stereogenic center, such as secondary alcohols, may be overcome by employing a so-called stereoinversion [66], which has only been achieved through biocatalytic methods. This technique consists in the transformation of a racemate S_R/S_S involving the enantio-specific stereochemical inversion of one enantiomer (S_S) by a chemically stable achiral (or prochiral) intermediate I (Scheme 2.1).

For instance, stereoinversion of *sec*-alcohols was achieved by employing whole cells [67–76] (Scheme 2.9). Although no definite proof exists, it was proposed by various groups that microbial stereoinversion occurs by an oxidation–reduction sequence. Thus, one enantiomer of a racemic mixture is selectively oxidized to the corresponding ketone under catalysis of a dehydrogenase, while the mirror-image counterpart remains unaffected. Then, the ketone is reduced again in a subsequent step by a different enzyme displaying opposite stereochemical preference. Overall, this two-step oxidation–reduction sequence constitutes formally a deracemization process. Due to the involvement of a consecutive oxidation–reduction reaction, the net redox balance of the process is zero and (in an ideal case) no external cofactor recycling is necessary since the redox equivalents, such as NAD(P)H, may be recycled internally between both steps, e.g., by using whole-cell systems.

The success of a biocatalytic stereoinversion by a redox process is determined by the following crucial point: for the entropy balance of the process, which is required to achieve a high optical purity of the product, at least one of the two redox reactions has to be irreversible [77,78]. The origin of this irreversibility is currently under investigation and the data available to date reveal a rather puzzling picture. For instance, deracemization of various terminal (\pm)-1,2-diols by the yeast *Candida parapsilosis* has been claimed to operate through an (*R*)-specific NAD⁺-linked dehydrogenase and an (*S*)-specific NADPH-dependent reductase. Although no detailed data were given, the latter step was proposed to be irreversible. Observations on the fungus *Geotrichum candidum* prove the requirement of



SCHEME 2.9 Deracemization of *sec*-alcohols based on a biocatalytic oxidation–reduction sequence.

molecular oxygen, which would suggest the involvement of an alcohol oxidase rather than an alcohol dehydrogenase.

Table 2.1 gives an overview of secondary alcohols that have been successfully deracemized by microbial stereoinversion employing various organisms. It is noteworthy that biocatalytic deracemization by stereoinversion of secondary alcohols was only accomplished by using whole cells.

Molecules possessing two chiral alcohol moieties were deracemized by one-step stereoinversion employing *Corynesporium cassiicola* and *Candida boidinii*: *rac*-1,2-indanediol and *rac*-1,2-cyclohexanediol were deracemized employing *C. cassiicola* to give the (*S,S*)-diol in >99% ee at up to 83% isolated yield [72,79]; employing *C. boidinii*, (*R,R*)-2,4-pentanediol was obtained from the racemate [73].

Stereoinversion was also observed for more complex molecules: one hydroxyl group of the two alcohol moieties of debenzylbutanolides, which possesses four chiral centers, was inverted by plant cells of *Catharanthus roseus* (periwinkle) to afford a product showing >99% diastereomeric excess [80].

Another example of a biocatalytic stereoinversion is the deracemization of α -chiral aryl- or aryloxy-carboxylic acids by isomerases [81]. Employing this methodology, deracemization of 2-aryl- and 2-aryloxy-propanoic acids [82,83] was performed using fermenting or resting cells of *Nocardia diaphanozonaria* JCM3208: 2-aryl- and *para*-substituted 2-aryloxy-propanoic acids were obtained in high yields with excellent ee from the corresponding racemates (Scheme 2.10) [84].

Mechanistically, this stereoinversion process was proposed to occur in the following sequence: (i) formation of an “activated” acyl-CoA-derivative of the (*S*)-acid; (ii) epimerization of the latter to yield the (*R*)-isomer; and finally (iii) hydrolysis of the (*R*)-acyl-CoA-ester. To eliminate the competing β -oxidation of the substrate, inhibitors of acyl-CoA-dehydrogenase, the first enzyme in the β -oxidation pathway, can be added [85].

Moreover, α -chiral carboxylic acids can also be stereoinverted employing an oxidase, namely glycolate oxidase, which oxidizes the (*S*)-enantiomer of the α -hydroxy acid to the keto-acid, which in turn is reduced in a second step by lactate dehydrogenase to the (*R*)-enantiomer, allowing to obtain enantiopure (*R*)- α -hydroxy acids (Scheme 2.11) [86,87]. A similar strategy with crude enzyme preparation as well as whole cells was employed for the conversion of racemic mandelic acid into its (*R*)-enantiomer [88,89].

An analogous combination of an oxidase (in this case a D-amino acid oxidase) and an amino acid dehydrogenase (leucine dehydrogenase) has been used to produce L-methionine from the racemate by enzymatic stereoinversion [90].

2.4 ENANTIOCONVERGENT PROCESSES

Deracemization may be achieved through so-called enantioconvergent processes, in such a way that each of the enantiomers is converted into the same product enantiomer P_R through two independent pathways (Scheme 2.1). Thus, whereas enantiomer S_R is reacted to product P_R through *retention* of configuration, its counterpart S_S is transformed with *inversion* of configuration. In general, both reactions are conducted in a stepwise fashion. The crucial prerequisites for the proper functioning of such systems are:

1. The first step must combine excellent enantiospecificity *and* stereospecificity with regard to retention or inversion. In other words, the requirements of the chiral recognition *and* stereochemistry of the respective transformation are high, and it is not surprising that these specificities are usually only achieved by enzymes.

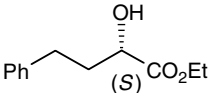
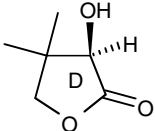
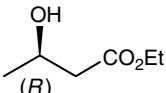
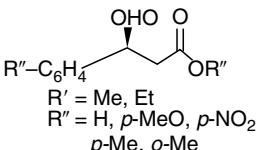
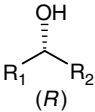
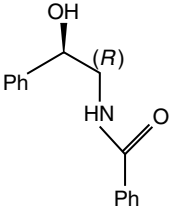
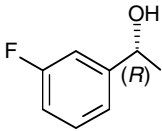
TABLE 2.1
Deracemization of *rac*-*sec*-Alcohols Bearing a Single Stereocenter by Microbial Stereoconversion

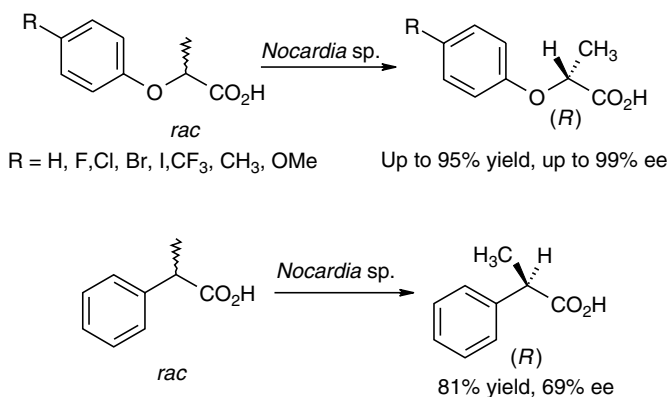
Product	Organism	ee max (%)	Conversion /yield (%)	Reference
	<i>Candida parapsilosis</i>	>99	85–90% yield	75
	<i>Rhodococcus erythropolis</i>	94	70% yield	134
	<i>Geotrichum candidum</i>	96	75% yield	78
<p> $R' = \text{Me, Et}$ $R'' = \text{H, } p\text{-MeO, } p\text{-NO}_2$ $p\text{-Me, } o\text{-Me}$ </p>	<i>Candida parapsilosis</i>	99	62–75% yield	135
<p> $R_1 = \text{Ar, Het-Ar, Ar-CH}_2\text{-}$ $R_2 = \text{Me, Et, } n\text{-hexyl, vinyl}$ </p>	<i>Sphingomonas paucimobilis</i>	99	up to 90% yield	136
	<i>Cunninghamella echinulata</i>	92	57% yield	137
	<i>Aspergillus terreus</i> CCT 3320	>99	59% yield	138

continued

TABLE 2.1 (continued)

Deracemization of *rac*-sec-Alcohols Bearing a Single Stereocenter by Microbial Stereoinversion

Product	Organism	ee max (%)	Conversion/yield (%)	Reference
	<i>Aspergillus terreus</i> CCT 4083	>99	Up to 86% conversion	139
	<i>Geotrichum candidum</i>	Up to 99	55–100% conversion	68, 140
	<i>Catharanthus roseus</i>	Up to >99	92–100% yield	70, 141
	<i>Rhizopus oryzae</i>	95–97 (<i>R</i>)	73–76% yield for (<i>R</i>)	142
	<i>Candida boidinii</i>	85 (<i>S</i>)	71% yield for (<i>S</i>)	143
$R_1 = \text{Ar, Het-Ar, Ar-CH}_2\text{-}$	<i>Pichia methanolica</i>	Up to 100	Up to 88% conversion	
$R_2 = \text{Me, Et, c-hexyl, vinyl}$	<i>Hansenula polymorpha</i>	Up to 60	Up to 100% conversion	
	<i>C. parapsilosis</i>	100, $n = 2, 3$ 79, $n = 1$	Up to 100% yield	77
	(<i>R</i>)-alcohol: <i>Nocardia fusca</i> , <i>N. globerulea</i> , <i>N. erythropolis</i> ; (<i>S</i>): <i>Nocardia pseudosporangifera</i>	100 (<i>R</i>) 98 (<i>S</i>)	83% conversion for (<i>R</i>) 70% conversion for (<i>S</i>)	76, 144

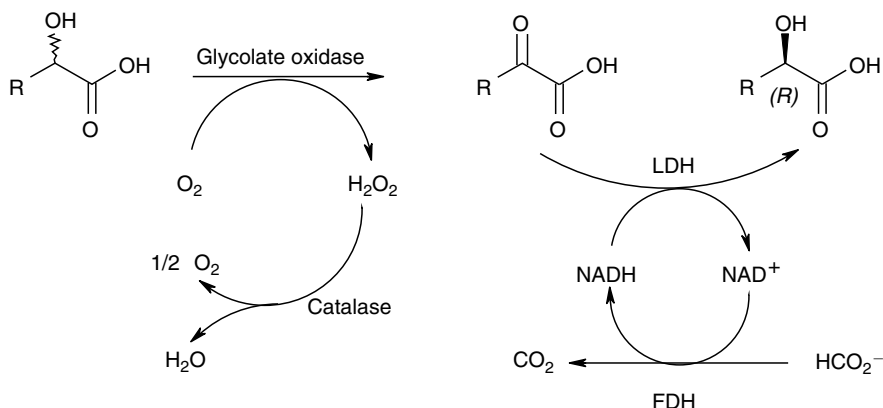


SCHEME 2.10 Deracemization of α -phenyl- and *p*-substituted α -aryloxypropanoic acids by enzymes of *Nocardia diaphanozonaria* JCM3208.

2. Since the starting material for the second step (i.e., S_S) is enantiomerically enriched (or even pure), only high stereospecificity is required with respect to inversion or retention of configuration. Hence, this step may also be performed by using a chemocatalyst.
3. An important factor for the economy of the whole process is the compatibility of the reaction conditions of both steps. If, for instance, the conditions are incompatible, separation of product P_R (formed during step 1 from S_R) from the remaining enantiomeric starting material S_S is required, which usually accompanies loss of material. After all, it appears anachronistic to separate materials from each other that are to be combined at the end of the process. Thus, successful enantioconvergent processes should always be performed in a one-pot fashion.

2.4.1 ENANTIOCONVERGENCE USING TWO BIOCATALYSTS

The only enzymes that may transform non-natural compounds with concomitant *inversion* of configuration during catalysis are: (i) glycosidases [91]; (ii) dehalogenases [92]; (iii) sulfatases [93]; and (iv) epoxide hydrolases [94]. Whereas glycosidases cannot be employed for

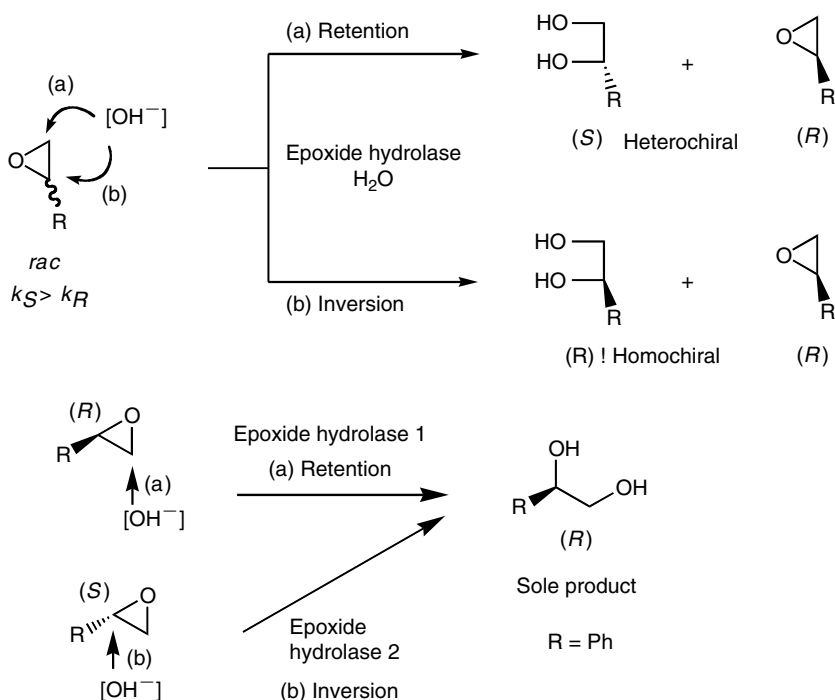


SCHEME 2.11 Glycolate oxidase/LDH-catalyzed stepwise deracemization of *rac*-2-hydroxy acids. LDH, lactate dehydrogenase; FDH, formate dehydrogenase.

deracemization, because their substrates are diastereomers rather than enantiomers, dehalogenases are not widely distributed in Nature and they exhibit a limited substrate tolerance. Sulfatases are under investigation. The application of aryl-sulfatases in preparative biotransformations is very limited [95], but alkyl sulfatases seem to be quite flexible in catalyzing the enantioselective hydrolysis of sulfate esters through inversion of configuration [96].

Epoxide hydrolases from microbial sources, such as bacteria, fungi, and (red) yeasts, have been shown to possess a great potential for the stereoselective hydrolysis of epoxides to furnish the corresponding vicinal diols [97–99]. In contrast to ester hydrolysis catalyzed by lipases, esterases, or proteases, where the absolute configuration at the stereogenic center(s) always remains the same throughout the reaction, enzymatic hydrolysis of epoxides may take place through an attack on either carbon atom of the oxirane ring, and it is the structure of the substrate and of the enzyme that determines the regioselectivity of the attack [100]. This is exemplified in Scheme 2.12. If the (*S*)-enantiomer is preferentially hydrolysed from the racemate with *retention* of configuration (pathway a), kinetic resolution furnishes a mixture of (*S*)-diol and unreacted (*R*)-epoxide. On the contrary, the corresponding (*R*)-diol is produced from the (*S*)-oxirane, if the enzyme acts with *inversion* of configuration (pathway b). Therefore, enantioconvergent hydrolysis of epoxides should be feasible when appropriate enzymes are available.

An elegant deracemization of (\pm)-styrene oxide was developed by making use of two epoxide hydrolase activities from fungal sources [101]. Whereas *Aspergillus niger* preferentially hydrolyzed the (*R*)-enantiomer with retention of configuration by producing (*R*)-phenylethan-1,2-diol (Scheme 2.12, epoxide hydrolase 1), *Beauveria bassiana* (formally denoted as *B. sulfurescens*) showed opposite enantiopreference, i.e., (*S*), with matching



SCHEME 2.12 Enzymatic hydrolysis of epoxides proceeding with retention or inversion of configuration.

opposite regioselectivity, causing inversion of configuration (epoxide hydrolase 2). The combination of both biocatalysts in a single reactor led to almost complete deracemization.

The same methodology has been employed to prepare an enantiopure building block of Eliprodil, a promising neuroprotective agent, using high substrate concentrations in a biphasic reactor and low temperatures, to minimize significant spontaneous hydrolysis [102].

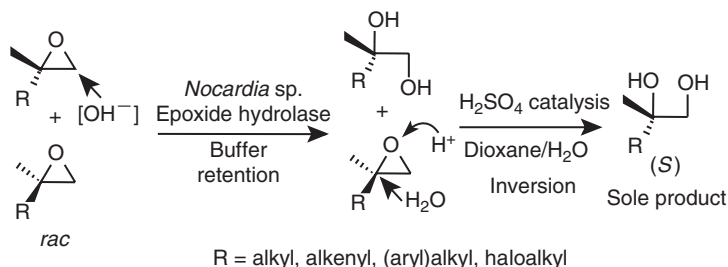
2.4.2 ENANTIOCONVERGENCE USING COMBINED BIO- AND CHEMOCATALYSIS

In order to transform both enantiomers obtained from lipase-catalyzed kinetic resolution of *sec*-alcohols by acyl-transfer or ester hydrolysis, the following one-pot two-step technique was employed [103–107]. Without separation, the mixture of *sec*-alcohol and the corresponding pseudo-enantiomeric carboxylic acid ester was subjected to chemical inversion of the alcohol by treatment with mesyl chloride or (for large-scale reactions) with fuming nitric acid under carefully controlled reaction conditions, yielding a mixture of enantiomeric activated and nonactivated esters. For small-scale reactions, Mitsunobu conditions may be likewise employed [108]. Both compounds were hydrolyzed by strong base with concurrent *inversion* and *retention* of configuration for the activated and nonactivated ester, respectively. As a consequence, a single enantiomeric *sec*-alcohol was formed as the sole product.

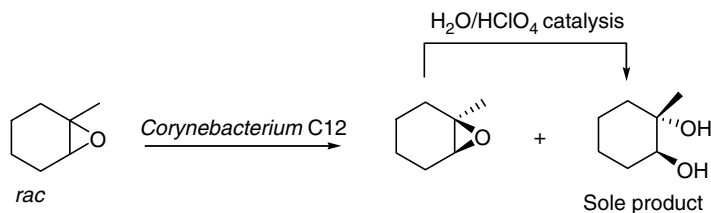
Bacterial epoxide hydrolases have been shown to be the biocatalysts of choice for the enantioselective hydrolysis of 2,2-disubstituted oxiranes, showing virtually absolute enantioselectivities ($E > 200$) [109]. In this case, the reaction proved to proceed invariably through *retention* of configuration. As the existence of enzymes that attack a quaternary carbon atom with *inversion* of configuration is rather unlikely, deracemization by a two-enzyme system as described above is impossible (see Scheme 2.12). However, the combination of bio- and chemocatalysis proved to be very efficient (Scheme 2.13) [110].

Thus, kinetic resolution of 2,2-disubstituted epoxides using a *Nocardia* sp. epoxide hydrolase proceeded with excellent enantio- and regioselectivity by furnishing the corresponding (*S*)-diol and (*R*)-epoxide in the first step. Then, the remaining epoxide was transformed by acid catalysis with *inversion* of configuration in the second step under carefully controlled reaction conditions to yield the corresponding (*S*)-diol in virtually enantiopure form and in high chemical yields (>90%). This methodology proved to be highly flexible and was also applicable to styrene-oxide type substrates [111].

In a related fashion, deracemization of *rac*-1-methyl-1,2-epoxycyclohexane was achieved by incubation with *Corynebacterium* sp. C12 and subsequent acid catalysis of the remaining epoxide (Scheme 2.14) [112]. Recently, a similar chemo-enzymatic procedure was reported using *Methylobacterium* sp. as biocatalyst [113]. Instead of the chemical transformation of the nonhydrolysed oxirane, the formed diol can be converted into the remaining epoxide [114].



SCHEME 2.13 Enantioconvergent hydrolysis of 2,2-disubstituted epoxides by combination of bio- and chemocatalysis.



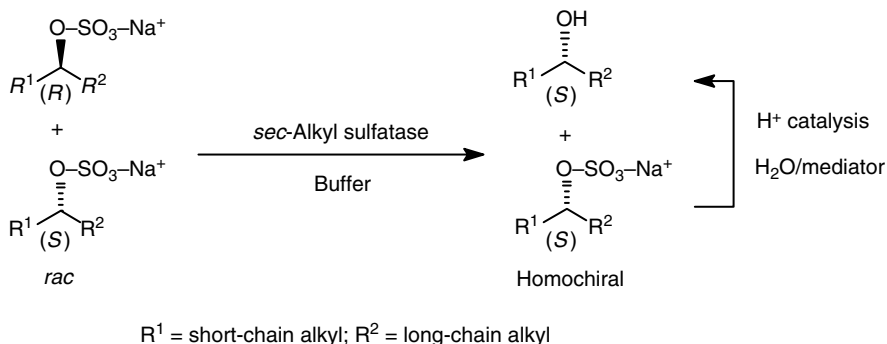
SCHEME 2.14 Deracemization of 1-methyl-1,2-epoxycyclohexane.

Enantioconvergent processes using a single biocatalyst can also be accomplished with certain sulfatases, since the stereochemical course of sulfate ester hydrolysis (i.e., *retention* vs. *inversion*) can be controlled by the choice of the appropriate subtype of this enzyme class. Thus, whereas aryl-sulfatases generally act through *retention* of configuration, some alkyl-sulfatases lead to *inversion*. Consequently, when a racemic sulfate ester is used as the substrate of these alkyl-sulfatases, both the formed product *sec*-alcohol and the remaining nonconverted sulfate ester possess the same absolute configuration, and hence constitute a homochiral product mixture (Scheme 2.15) [115]. Removal of the sulfate ester group from the remaining nonhydrolyzed (*S*)-sulfate ester with retention of configuration was achieved under acid catalysis [116] and led to the formation of the corresponding (*S*)-*sec*-alcohol as the sole product.

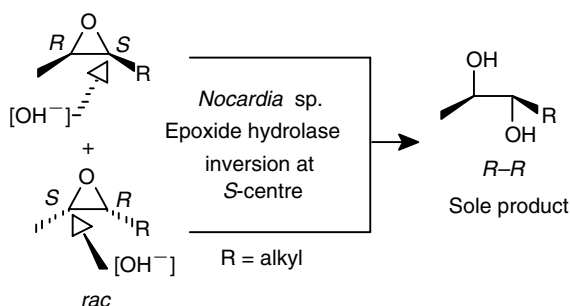
Although the *stereoselectivities* of a *sec*-alkyl sulfatase RS2 from *Rhodococcus* sp. were absolute with respect to inversion, *enantioselectivities* ranged from low to moderate (*E*-values up to 21). The latter values could be improved by using low concentrations of Fe^{3+} acting as enantioselective inhibitor [117]. Highly enantioselective sulfatases were recently identified in hyperthermophilic *Archaea*, such as *Sulfolobus* sp. [118].

2.4.3 ENANTIOCONVERGENCE USING A SINGLE BIOCATALYST

Processes depending on more than one catalyst are generally sensitive with respect to the kinetic tuning of both reactions and, therefore, enantioconvergent reactions that depend on a *single* catalyst would be more reliable in practice. However, the requirements for this single catalyst are extremely high, i.e., it has to exhibit not only high *enantioselectivity*, but also show at the same time *opposite regioselectivity* for the transformation of each enantiomer in order



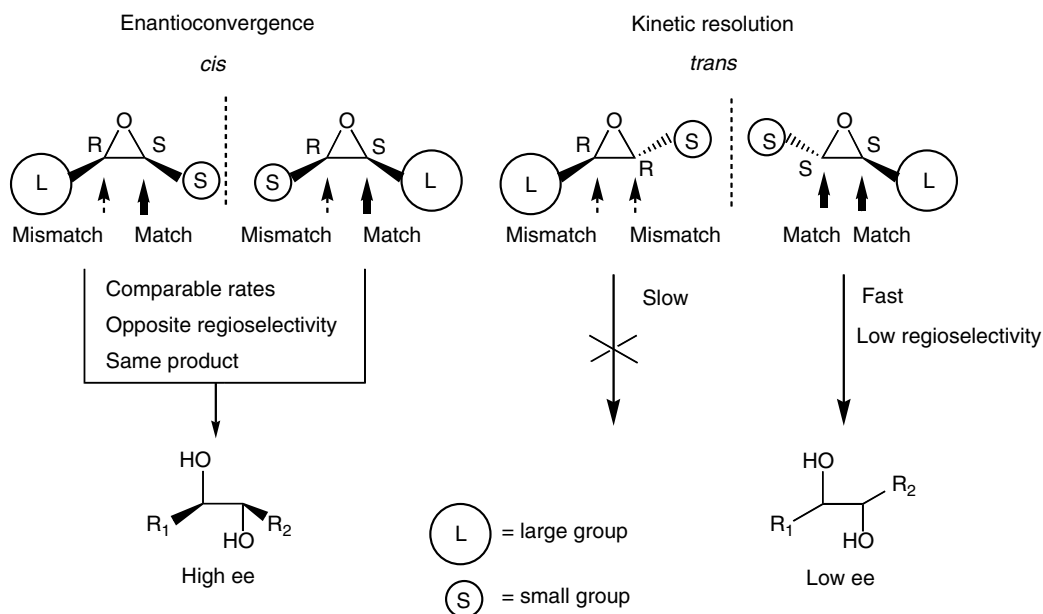
SCHEME 2.15 Chemo-enzymatic deracemization of *sec*-alcohols by their corresponding sulfate esters using inverting *sec*-alkyl sulfatases.



SCHEME 2.16 Enantioconvergent hydrolysis of 2,3-disubstituted epoxides using a single biocatalyst.

to make the overall process enantioconvergent. Therefore, such processes catalyzed by a single (bio)catalyst are very rare (Scheme 2.16) [119,120]. For instance, an epoxide hydrolase from *Nocardia* sp. hydrolyzed both enantiomers of *cis*-2,3-disubstituted epoxides with opposite *regioselectivity* by attack at their respective (*S*)-oxirane carbon atom with strict inversion of configuration, yielding the corresponding (*R,R*)-diol as the sole product in up to 92% ee and 85% chemical yield [121].

Detailed analysis of this system using a range of 2,3-disubstituted *rac*-oxiranes revealed that the relative *cis*- or *trans*-configuration was of critical importance for the enantioconvergent microbial biohydrolysis (Scheme 2.17) [122]. Due to the fact that each enantiomer of a *cis*-configured *rac*-epoxide possesses a single (*S*)-configured carbon atom (match), both enantiomers are hydrolyzed at comparable rates and with opposite regioselectivity, leading to the same stereoisomeric product. In contrast, in the *trans*-series, one enantiomer (possessing two “matches”) is hydrolyzed very fast with low regioselectivity, whereas the mirror-image



SCHEME 2.17 Relative stereochemistry of 2,3-disubstituted oxiranes determines enantioconvergence or kinetic resolution.

counterpart remains unaffected as the result of two “mismatches.” Consequently, kinetic resolution leads to the formation of the corresponding diol in low ee

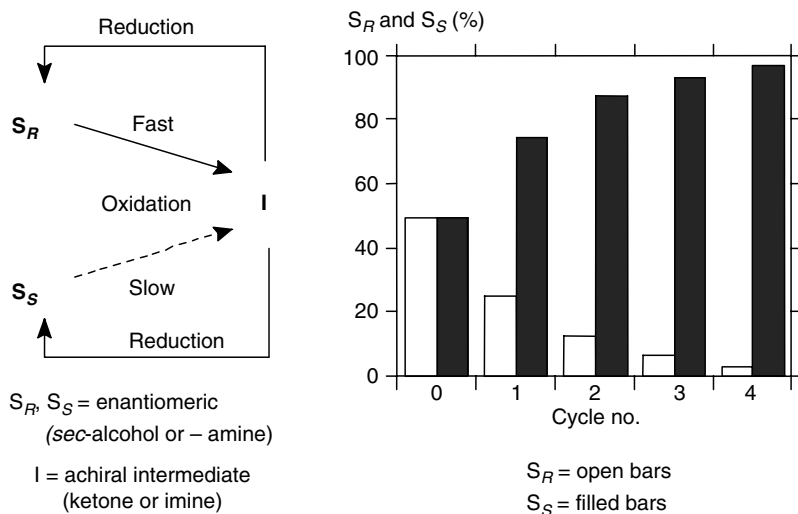
2.5 DERACEMIZATION BY A CYCLIC OXIDATION–REDUCTION SEQUENCE

Deracemization of compounds bearing a chiral *sec*-hydroxyl or amino group can be achieved by a novel process based on a cyclic oxidation–reduction sequence [123,124]. The system consists of two independent reactions outlined in Scheme 2.18.

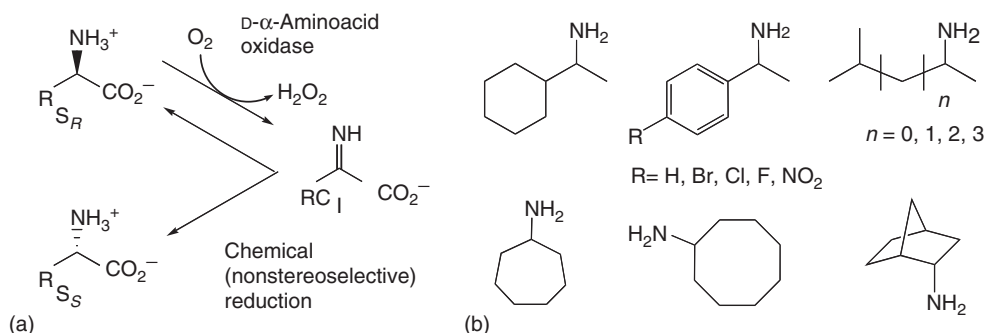
First, one enantiomer of the secondary alcohol or amine (S_R) is selectively oxidized from the starting racemate ($S_R + S_S$) to yield the achiral intermediate I, i.e., the corresponding ketone or imine, respectively. Then, the product is chemically reduced in a nonselective fashion to yield again a racemic mixture. Both reactions alone are of limited use for the preparation of enantiopure material, since step 1 (i.e., a kinetic resolution by enantioselective oxidation) is limited to a 50% theoretical yield of chiral nonreacting substrate enantiomer S_S and achiral intermediate I, and step 2 does not show any chiral induction at all. However, combination of both steps in a cyclic mode leads to a highly versatile deracemization technique.

The functioning of this system is explained by the following example. If the selectivity of step 1 is assumed to be absolute, only S_R is selectively oxidized to form achiral I in 50% yield by leaving S_S untouched. In the second step, intermediate I is nonselectively reduced to furnish $S_R + S_S$ in equal amounts of 25% each. As a consequence, the enantiomeric composition of S_R/S_S after a single cycle now equals 25/75. The diagram shown in Scheme 2.18 reveals that further cycles lead to a gradual increase of enantiomer S_S at the expense of S_R , and that the enantiomeric excess of the substrate is already well above 90% after only four cycles, assuming absolute enantioselectivity. Overall, if the cyclic process is driven in the forward direction, enantiomer S_S represents the “sink” of material in the whole system.

For practical applications, however, enantioselectivities in step 1 often range below E -values of 100. For these cases, the enantioselectivity determines two crucial factors of the



SCHEME 2.18 Deracemization of *sec*-alcohols and -amines by a cyclic oxidation–reduction sequence.



SCHEME 2.19 (a) Deracemization of *rac*- α -amino acids using enantioselective bio-oxidation coupled to nonselective chemical reduction of the imino acid intermediate I. (b) Selection of chiral amines that can be oxidized by an Asn336Ser variant of the amine oxidase from *Aspergillus niger*.

system: (i) the maximum obtainable ee at equilibrium; and (ii) the number of cycles that are required to reach this value. The merits and limits of cyclic deracemization systems have been described based on the underlying kinetics [123,124].

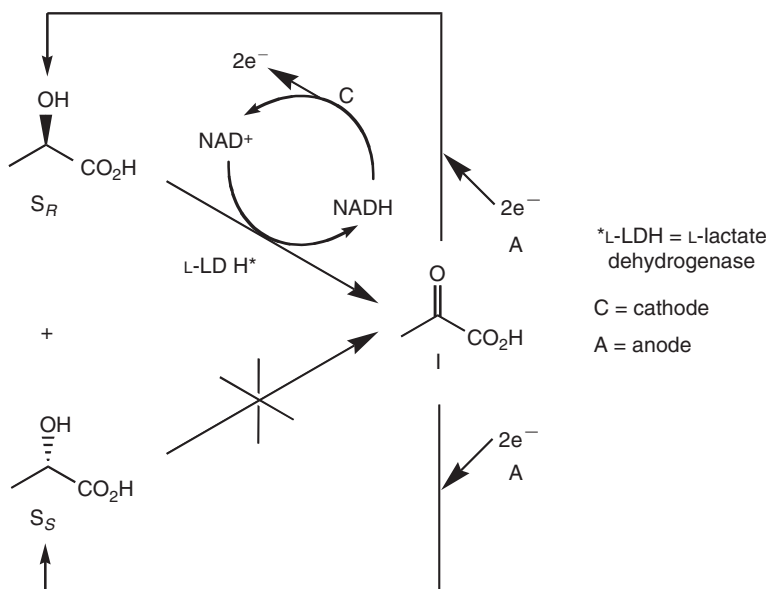
This procedure has been employed for the deracemization of α -amino acids by (i) coupling an enantioselective oxidation (catalyzed by a D-specific amino acid oxidase, producing the corresponding nonchiral imino acid as a short-lived intermediate) to a (ii) chemical non-selective reduction step, e.g., with sodium borohydride or amine borane (Scheme 2.19) [125–128]. Overall, this process led to the formation of L-amino acids from the racemate [123,124] and has been used for the resolution of racemic pipecolic acid, an L-proline homolog [129].

In order to extend the applicability of this elegant process to chiral amines (which are not oxidized by amino acid oxidases), an amine oxidase from *A. niger* (predominantly acting on α -methylbenzyl amine) was identified as a suitable biocatalyst. Directed evolution of this enzyme resulted in an amine oxidase possessing not only a wide substrate spectrum but also good enantioselectivity [130–132]. The Asn336Ser variant of the amine oxidase showed highest activity toward substrates bearing a methyl substituent and a bulky alkyl/aryl group adjacent to the amino-carbon atom (Scheme 2.18). In all cases examined so far, the enzyme variant was enantioselective for the (*S*)-isomer of the *rac*-amine substrate. For large-scale applications, improvements of enzyme stability, activity, and selectivity are still required. The deracemization of chiral amines should now be feasible by using this enzyme.

A related approach is based on the electro- and biochemical oxidation and/or reduction of lactate involving the recycling of NADH [133]. In this way, complete inversion of L-lactate into D-lactate was achieved in a model reactor (Scheme 2.20).

2.6 SUMMARY

The development of methods for the preparation of chiral compounds in 100% chemical and optical yields from racemates is one of the current challenges in asymmetric synthesis. Several approaches have been described so far, which are either based on modifications of classic kinetic resolution, such as re-racemization and repeated resolution or dynamic resolution, or on the transformation of enantiomers through enantioconvergent pathways, which is usually achieved by combination of chemo- and/or biocatalysts in sequential reactions or—most elegantly—even by a single (bio)catalyst. Finally, cyclic deracemization and stereoinversion through an oxidation–reduction sequence are feasible options. It has to be emphasized,



SCHEME 2.20 Deracemization of α -hydroxyacid through a bio- and electrochemical cyclic oxidation–reduction sequence.

however, that each of the above-described approaches offers a solution for only certain types of stereochemical problems and the corresponding substrate classes, but none of the methods can be employed as a general solution.

ACKNOWLEDGMENT

Financial support by Fundación Ramón Areces is gratefully acknowledged.

REFERENCES

1. Stinson, S.C., *Chem. Eng. News.*, Sept 28, 46–78, 1992.
2. Sheldon, R.A., *Chirotechnology*, Marcel Dekker, New York, 1993.
3. Collins, A.N., Sheldrake, G.N., and Crosby, J., Eds., *Chirality in Industry*, vols. I and II, Wiley, Chichester, 1992 and 1997.
4. Faber, K., *Biotransformations in Organic Chemistry*, 5th ed., Springer, Heidelberg, 2004.
5. Drauz, K. and Waldmann, H., *Enzyme Catalysis in Organic Synthesis*, 2nd ed., vols. I and II, VCH, Weinheim, Germany, 2002.
6. Liese, A., Seelbach, K., and Wandrey, C., *Industrial Biotransformations*, VCH, Weinheim, Germany, 2000.
7. Bornscheuer, U.T. and Kazlauskas, R.J., *Hydrolases in Organic Synthesis*, VCH, Weinheim, Germany, 1999.
8. Schoffers, E., Golebiowski, A., and Johnson, C.R., *Tetrahedron*, 52, 3769–3826, 1996.
9. Kagan, H.B. and Fiaud, J.C., *Topics Stereochem.*, 18, 249–330, 1988.
10. Horeau, A., *Tetrahedron*, 31, 1307–1309, 1975.
11. Sih, C.J. and Wu, S.H., *Topics Stereochem.*, 19, 63–125, 1989 (for biocatalyzed reactions).
12. Martin, V.S., Woodard, S.S., Katsuki, T., Yamada, Y., Ikeda, M., and Sharpless, K.B., *J. Am. Chem. Soc.*, 103, 6237–6240, 1981 (for nonbiocatalyzed reactions).
13. Balavoine, G., Moradpour, A., and Kagan, H.B., *J. Am. Chem. Soc.*, 96, 5152–5158, 1974 (for prebiotic reactions).

14. Faber, K., and Kroutil, W., ~13.000 entries, May 2006.
15. Faber, K., *Indian J. Chem. Sect. B*, 31, 921–924, 1992.
16. Cotterill, I.C., Jaouhari, R., Dorman, G., Roberts, S.M., Scheinmann, F., and Wakefield, B.J., *J. Chem. Soc. Perkin Trans.*, 1, 2505–2512, 1991.
17. Chen, C.S., Fujimoto, Y., Girdaukas, G., Sih, C.J., *J. Am. Chem. Soc.*, 104, 7294–7299, 1982.
18. Azerad, A.R. and Buison, D., *Curr. Opin. Biotechnol.*, 11, 565–571, 2000.
19. Stecher, H. and Faber, K., *Synthesis*, 1–16, 1997.
20. Strauss, U.T., Felfer, U., and Faber, K., *Tetrahedron Asymmetry*, 10, 107–117, 1999.
21. Faber, K., *Chem. Eur. J.*, 7, 5004–5010, 2001.
22. Xie, Y.C., Liu, H.Z., and Chen, J.Y., *Biotechnol. Lett.*, 20, 455–458, 1998.
23. Kamphuis, J., Boesten, W.H.J., Kaptein, B., Hermes, H.F.M., Sonke, T., Broxterman, Q.B., van den Tweel, W.J.J., and Schoemaker, H.E., The production and uses of optically pure natural and unnatural amino acids, in *Chirality in Industry*, Collins, A.N., Sheldrake, G.N., and Crosby, J., Eds., Wiley, New York, 1992, pp. 187–208.
24. Eliel, E.L. and Wilen, S.H., *Stereochemistry of Organic Compounds*, Wiley, New York, 1994.
25. Ebbers, E.J., Ariaans, G.J.A., Houbiers, J.P.M., Bruggink, A., and Zwanenburg, B., *Tetrahedron*, 53, 9417–9476, 1997.
26. Um, P.J. and Drueckhammer, D.G., *J. Am. Chem. Soc.*, 120, 5605–5610, 1998.
27. Huerta, F.F., Minidis, A.B.E., and Bäckvall, J.E., *Chem. Soc. Rev.*, 30, 321–331, 2001.
28. Larsson, A.L.E., Persson, B.A., and Bäckvall, J.E., *Angew. Chem. Int. Ed. Engl.*, 36, 1211–1212, 1997.
29. Allen, J.V. and Williams, J.M.J., *Tetrahedron Lett.*, 37, 1859–1862, 1996.
30. Palmer, M.J. and Wills, M., *Tetrahedron Asymmetry*, 10, 2045–2061, 1999.
31. Pàmies, O. and Bäckvall, J.E., *Chem. Eur. J.*, 7, 5052–5058, 2001.
32. Samec, J.S.M. and Bäckvall, J.E., *Chem. Eur. J.*, 8, 2955–2961, 2002.
33. Èll, A.H., Samec, J.S.M., Brasse, C., and Bäckvall, J.E., *Chem. Commun.*, 1144–1145, 2002.
34. Granberg, K.L. and Bäckvall, J.E., *J. Am. Chem. Soc.*, 114, 6858–6863, 1992.
35. Broxterman, Q.B., Boesten, W.H.J., Kamphuis, J., Kloosterman, M., Meijer, E.M., and Schoemaker, H.E., Chemo-enzymatic production methods for optically pure intermediates of potential commercial interest, in *Opportunities in Biotransformations*, Copping, L.G., Martin, R.E., Pickett, J.A., Bucke, C., and Bunch, A.W., Eds., Elsevier, London, 1990, pp. 148–169.
36. Wolf, L.B., Sonke, T., Tjen, K.C.M.F., Kaptein, B., Broxterman, Q.B., Schoemaker, H.E., and Rutjes F.P.J.T., *Adv. Synth. Catal.*, 343, 662–674, 2001.
37. Adams, E., *Adv. Enzymol. Relat. Areas. Mol. Biol.*, 44, 69–138, 1976.
38. Gallo, K.A., Tanner, M.E., and Knowles, J.R., *Biochemistry*, 32, 3991–3997, 1993.
39. Kenyon, G.L., Gerlt, J.A., and Kozarich, J.W., *Acc. Chem. Res.*, 28, 178–186, 1995.
40. Felfer, U., Goriup, M., Koegl, M.F., Wagner, U., Larissegger-Schnell, B., Faber, K., and Kroutil, W., *Adv. Synth. Catal.*, 347, 951–961, 2005.
41. Goriup, M., Strauss, U.T., and Faber, K., *J. Mol. Catal. B Enzym.*, 15, 207–211, 2001.
42. Felfer, U., Strauss, U.T., Kroutil, W., Fabian, W.M.F., and Faber, K., *J. Mol. Catal. B Enzym.*, 15, 213–222, 2001.
43. Hegeman, G.D., Rosenberg, E.Y., and Kenyon, G.L., *Biochemistry*, 9, 4029–4035, 1970.
44. Strauss, U.T., and Faber, K., *Tetrahedron Asymmetry*, 10, 4079–4081, 1999.
45. *Pogorevc, M., Stecher, H., and Faber, K., *Biotechnol. Lett.*, 24, 857–860, 2002.
46. Schleifer, K.H. and Kandler, O., *Bacteriol. Rev.*, 36, 407–477, 1972.
47. Bycroft, B.W., *Dictionary of Antibiotics and Related Substrates*, Chapman & Hall, New York, 1988.
48. Yamada, H., Shimizu, S., Shimada, H., Tani, Y., Takahashi, S., and Ohashi, T., *Biochimie*, 62, 395–399, 1980.
49. Sylđatk, C., Müller, R., Pietzsch, M., and Wagner, F., Microbial and enzymatic production of L-amino acids from DL-monosubstituted hydantoins, in *Biocatalytic Production of Amino Acids and Derivatives*, Rozell, J.D. and Wagner, F., Eds., Hanser Publishers, New York, 1992, pp. 129–176.

*Mandelate racemase was found to be inactive in various organic solvent systems.

50. Pietzsch, M., Syldatk, C., and Wagner, F., *Ann. N. Y. Acad. Sci.*, 672, 478–483, 1992.
51. Ward, R.S., *Tetrahedron Asymmetry*, 6, 1475–1490, 1995.
52. Caddick, S. and Jenkins, K., *Chem. Soc. Rev.*, 25, 447–456, 1996.
53. Noyori, R., Tokunaga, M., and Kitamura, M., *Bull. Chem. Soc. Jpn.*, 68, 36–56, 1995.
54. Kitamura, M., Tokunaga, M., and Noyori, R., *J. Am. Chem. Soc.*, 115, 144–152, 1993.
55. Kitamura, M., Tokunaga, M., and Noyori, R., *Tetrahedron*, 49, 1853–1860, 1993.
56. Um, P.J. and Drueckhammer, D.G., *J. Am. Chem. Soc.*, 120, 5605–5610, 1998.
57. Fülling, G. and Sih, C.J., *J. Am. Chem. Soc.*, 109, 2845–2846, 1987.
58. Inagaki, M., Hiratake, J., Nishioka, T., and Oda, J., *J. Am. Chem. Soc.*, 113, 9360–9361, 1991.
59. Brinksma, J., van der Deen, H., van Oeveren, A., and Feringa, B.L., *J. Chem. Soc. Perkin. Trans.*, 1, 4159–4163, 1998.
60. Allen, J.V., and Williams, J.M.J., *Tetrahedron Lett.*, 37, 1859–1862, 1996.
61. Larsson, A.L.E., Persson, B.A., and Bäckvall, J.E., *Angew. Chem.*, 109, 1256–1258, 1997.
62. Reetz, M.T. and Schimossek, K., *Chimia*, 50, 668–669, 1996.
63. Kitamura, M., Tokunaga, M., and Noyori, R., *J. Am. Chem. Soc.*, 115, 144–152, 1993.
64. Cecere, F., Galli, G., Della Penna, G., and Rappuoli, B., Ger Offen 2621076, addition to Ger Offen 2, 422,737, 1976; *Chem. Abst.*, 86, 107042t, 1976.
65. May, O., Verseck, S., Bommarius, A., and Drauz, K., *Org. Process Res. Dev.*, 6, 452–457, 2002.
66. Carnell, A.J., *Adv. Biochem. Eng. Biotechnol.*, 63, 57–72, 1999.
67. Buisson, D., Azerad, R., Sanner, C., and Larcheveque, M., *Biocatalysis*, 5, 249–265, 1992.
68. Nakamura, K., Inoue, Y., Matsuda, T., and Ohno, A., *Tetrahedron Lett.*, 36, 6263–6266, 1995.
69. Fantin, G., Fogagnolo, M., Giovannini, P.P., Medici, A., and Pedrini, P., *Tetrahedron Asymmetry*, 6, 3047–3053, 1995.
70. Takemoto, M. and Achiwa, K., *Tetrahedron Asymmetry*, 6, 2925–2928, 1995.
71. Tsuchiya, S., Miyamoto, K., and Ohta, H., *Biotechnol. Lett.*, 14, 1137–1142, 1992.
72. Carnell, A.J., Iacazio, G., Roberts, S.M., and Willetts, A.J., *Tetrahedron Lett.*, 35, 331–334, 1994.
73. Matsumura, S., Kawai, Y., Takahashi, Y., and Toshima, K., *Biotechnol. Lett.*, 16, 485–490, 1994.
74. Shimizu, S., Hattori, S., Hata, H., and Yamada, H., *Enzyme. Microb. Technol.*, 9, 411–416, 1987.
75. Chadha, A. and Baskar, B., *Tetrahedron Asymmetry*, 13, 1461–1464, 2002.
76. Ogawa, A.J., Xie, S.-X., and Shimizu, S., *Biotechnol. Lett.*, 21, 331–335, 1999.
77. Hasegawa, J., Ogura, M., Tsuda, S., Maemoto, S., Kutsuki, H., and Ohashi, T., *Agric. Biol. Chem.*, 54, 1819–1827, 1990.
78. Azerad, R. and Buisson, D., Stereocontrolled reduction of β -ketoesters with *Geotrichum candidum*, in *Microbial Reagents in Organic Synthesis*, S. Servi, Ed., NATO ASI Series C, Kluwer, Dordrecht, vol. 381, 1992, pp. 421–440.
79. Bulman Page P.C., Carnell, A.J., and McKenzie, M.J., *Synlett*, 774–776, 1998.
80. Takemoto, M., Matsuoka, Y., Achiwa, K., and Kutney, J.P., *Tetrahedron Lett.*, 41, 499–502, 2000.
81. Faber, K. and Kroutil, W., *Curr. Opin. Chem. Biol.*, 9, 181–187, 2005.
82. Kato, D., Mitsuda, S., and Ohta, H., *Org. Lett.*, 4, 371–373, 2002.
83. Mitsukura, K., Yoshida, T., and Nagasawa, T., *Biotechnol. Lett.*, 24, 1615–1621, 2002.
84. Kato, D., Mitsuda, S., and Ohta, H., *J. Org. Chem.*, 68, 7234–7242, 2003.
85. Kato, D., Miyamaoto, K., and Ohta, H., *Tetrahedron Asymmetry*, 15, 2965–2973, 2004.
86. Adam, W., Lazarus, M., Boss, B., Saha-Möller, C.R., Humpf, H.U., and Schreier, P., *J. Org. Chem.*, 62, 7841–7843, 1997.
87. Adam, W., Lazarus, M., Saha-Möller, C.R., and Schreier, P., *Tetrahedron Asymmetry*, 9, 351–355, 1998.
88. Takahashi, E., Nakamichi, K., and Furui, M., *J. Ferment. Bioeng.*, 80, 247–250, 1995.
89. Tsuchiya, S., Miyamoto, K., and Ohta, H., *Biotechnol. Lett.*, 14, 1137–1142, 1992.
90. Nakajima, N., Esaki, N., and Soda, K., *J. Chem. Soc. Chem. Commun.*, 947–948, 1990.
91. Sinnott, M.L., *Chem. Rev.*, 90, 1171–1202, 1990.
92. Leisinger, T. and Bader, R., *Chimia*, 47, 116–121, 1993.
93. Roy, A.B., *The Enzymes*, 5, 1–19, 1971.
94. Orru, R.V.A., Archelas, A., Furstoss, R., and Faber, K., *Adv. Biochem. Eng. Biotechnol.*, 63, 145–167, 1999.

95. Pelsy, G. and Klibanov, A.M., *Biotechnol. Bioeng.*, 25, 919–928, 1983.
96. Wallner, S.R., Pogorevc, M., Trauthwein, H., and Faber, K., *Eng. Life Sci.*, 4, 512–516, 2004.
97. Faber, K., Mischitz, M., and Kroutil, W., *Acta Chem. Scand.*, 50, 249–258, 1996.
98. Archelas, A. and Furstoss, R., *Annu. Rev. Microbiol.*, 51, 491–525, 1997.
99. Archer, I.V.J., *Tetrahedron*, 53, 15617–15662, 1997.
100. Mischitz, M., Mirtl, C., Saf, R., and Faber, K., *Tetrahedron Asymmetry*, 7, 2041–2046, 1996.
101. Pedragosa-Moreau, S., Archelas, A., and Furstoss, R., *J. Org. Chem.*, 58, 5533–5536, 1993.
102. Manoj, K.M., Archelas, A., Baratti, J., and Furstoss, R., *Tetrahedron*, 57, 695–701, 2001.
103. Danda, H., Nagatomi, T., Maehara, A., and Umemura, T., *Tetrahedron*, 47, 8701–8716, 1991.
104. Lemke, K., Ballschuh, S., Kunath, A., and Theil, F., *Tetrahedron Asymmetry*, 8, 2051–2055, 1997.
105. Vantinen, E. and Kanerva, L.T., *Tetrahedron Asymmetry*, 6, 1779–1786, 1995.
106. Takano, S., Suzuki, M., and Ogasawara, K., *Tetrahedron Asymmetry*, 4, 1043–1046, 1993.
107. Mitsuda, S., Umemura, T., and Hirohara, H., *Appl. Microbiol. Biotechnol.*, 29, 310–315, 1988.
108. Steinreiber, A., Stadler, A., Mayer, S.F., Faber, K., and Kappe, C.O., *Tetrahedron Lett.*, 42, 6283–6286, 2001.
109. Mischitz, M., Kroutil, W., Wandel, U., and Faber, K., *Tetrahedron Asymmetry*, 6, 1261–1272, 1995.
110. Orru, R.V.A., Mayer, S.F., Kroutil, W., and Faber, K., *Tetrahedron*, 54, 859–874, 1998.
111. Pedragosa-Moreau, S., Morisseau, C., Baratti, J., Zylber, J., Archelas, A., and Furstoss, R., *Tetrahedron*, 53, 9707–9714, 1997.
112. Archer, I.V.J., Leak, D.J., and Widdowson, D.A., *Tetrahedron Lett.*, 37, 8819–8822, 1996.
113. Uberbacher, B.J., Osprian I., Mayer S.F., and Faber, K., *Eur. J. Org. Chem.*, 7, 1266–1270, 2005.
114. Monfort, N., Archelas, A., and Furstoss, R., *Tetrahedron*, 60, 601–605, 2004.
115. Pogorevc, M., Kroutil, W., Wallner, S.M., and Faber, K., *Angew. Chem. Int. Ed. Engl.*, 41, 4052–4054, 2002.
116. Wallner, S.R., Nestl, B., and Faber, K., *Tetrahedron*, 61, 1517–1521, 2005.
117. Pogorevc, M., Strauss, U.T., Riermeier, T., and Faber, K., *Tetrahedron Asymmetry*, 13, 1443–1447, 2002.
118. Wallner, S.R., Nestl, B.M., and Faber, K., *Org. Lett.*, 6, 5009–5010, 2004.
119. Pedragosa-Moreau, S., Archelas, A., and Furstoss, R., *Tetrahedron*, 52, 4593–4606, 1996.
120. Bellucci, G., Chiappe, C., and Cordoni, A., *Tetrahedron Asymmetry*, 7, 197–202, 1996.
121. Kroutil, W., Mischitz, M., and Faber, K., *J. Chem. Soc. Perkin. Trans.*, 1, 3629–3636, 1997.
122. Mayer, S.F., Steinreiber, A., Orru, R.V.A., and Faber, K., *Eur. J. Org. Chem.*, 4537–4542, 2001.
123. Kroutil, W. and Faber, K., *Tetrahedron Asymmetry*, 9, 2901–2913, 1998.
124. Free shareware programs (Cyclo) running under Windows and Macintosh are available at <<http://borgel85.kfunigraz.ac.at>> or directly from the authors (W Kroutil, A Kleewein, K Faber  1998). A description of how to use the program is given in the help-file that accompanies the program.
125. Huh, J.W., Yokoigawa, K., Esaki, N., and Soda, K., *J. Ferment. Bioeng.*, 74, 189–190, 1992.
126. Soda, K., Oikawa, T., and Yokoigawa, K., *J. Mol. Catal. B Enzym.*, 11, 149–153, 2001.
127. Alexandre, F.R., Pantaleone, D.P., Taylor, P.P., Fotheringham, I.G., Ager, D.J., and Turner, N.J., *Tetrahedron Lett.*, 43, 707–710, 2002.
128. Turner, N.J., *Curr. Opin. Chem. Biol.*, 8, 114–119, 2004.
129. Huh, J.W., Yokoigawa, K., Esaki, N., and Soda, K., *Biosci. Biotech. Biochem.*, 56, 2081–2082, 1992.
130. Alexeeva, M., Enright, A., Dawson, M.J., Mahmoudian, M., and Turner, N.J., *Angew. Chem. Int. Ed. Engl.*, 41, 3177–3180, 2002.
131. Carr, R., Alexeeva, M., Enright, A., Eve, T.S.C., Dawson, M.J., and Turner, N.J., *Angew. Chem. Int. Ed. Engl.*, 42, 4807–4810, 2003.
132. Alexeeva, M., Carr, R., and Turner, N.J., *Org. Biomol. Chem.*, 1, 4133–4137, 2003.
133. Biade, A.E., Bourdillon, C., Laval, J.M., Mairesse, G., and Moiroux, J., *J. Am. Chem. Soc.*, 114, 893–899, 1992.
134. Shimizu, S., Hattori, S., Hata, H., Yamada, H., *Appl. Environ. Microbiol.*, 53, 519–522, 1987.
135. Padhi, S.K., Pandian, N.G., and Chadha, A., *J. Mol. Catal. B Enzym.*, 29, 25–29, 2004.
136. Allan, G.R. and Carnell, A.J., *J. Org. Chem.*, 66, 6495–6497, 2001.
137. Cardus, G.J., Carnell, A.J., Trauthwein, H., and Riermeier, T., *Tetrahedron Asymmetry*, 15, 239–243, 2004.

138. Comasseto, J.V., Omori, A.T., Andrade, L.H., and Porto, A.L.M., *Tetrahedron Asymmetry*, 14, 711–715, 2003.
139. Comasseto, J.V., Andrade, L.H., Omori, A.Z., Assis, L.F., and Porto, A.L.M., *J. Mol. Catal. B Enzym.*, 29, 55–61, 2004.
140. Nakamura, K., Fujii, M., and Ida, Y., *Tetrahedron Asymmetry*, 12, 3147–3153, 2001.
141. Takemoto, M. and Achiwa, K., *Chem. Pharm. Bull.*, 46, 577–580, 1998.
142. Demir, A.S., Hamamci, H., Sesenoglu, O., Neslihanoglu, R., Asikoglu, B., and Capanoglu, D., *Tetrahedron Lett.*, 43, 6447–6449, 2002.
143. Goswami, A., Mirfakhrae, K.D., and Patel, R.N., *Tetrahedron Asymmetry*, 10, 4239–4244, 1999.
144. Xie, S.-X., Ogawa, J., and Shimizu, S., *Appl. Microbiol. Biotechnol.*, 52, 327–331, 1999.

3 A Decade of Biocatalysis at Glaxo Wellcome

Mahmoud Mahmoudian

CONTENTS

3.1	Introduction	53
3.2	β -Lactam Antibiotics	56
3.3	Antileukemic Agents	58
3.4	Anti-Inflammatory Agents	61
3.5	Antiviral Agents	64
3.5.1	Epivir (Lamivudine)	64
3.5.2	Abacavir (Ziagen)	69
3.5.3	Zanamavir (Relenza)	71
3.5.4	Carbocyclic Nucleosides	75
3.5.4.1	Resolution of Intermediates to Carbocyclic Nucleosides <i>c</i> -BVdU and <i>c</i> -dG	76
3.5.4.2	Aristeromycin and Carbovir (<i>c</i> -d4G)	78
3.6	Enabling Technologies	80
3.6.1	Chiral Amines	80
3.6.1.1	Resolution of Amines by Substrate Engineering	83
3.6.1.2	Deracemization of Amino Acids and Amines	85
3.6.2	High-Throughput Screening Using Infrared Thermography	91
3.7	Summary	94
	Acknowledgments	96
	References	97

3.1 INTRODUCTION

The biotech industry has evolved into three major areas of application: (1) “red biotechnology” (pharmaceutical, therapeutic/medical applications); (2) “green biotechnology” (agricultural applications); and (3) “white biotechnology” (industrial applications). White biotechnology is at a relatively early stage in the chemical industry, but is seen as a potential key driver to the industry’s future. Industrial applications of biotechnology today include biofeedstocks that replace fossil fuel with sugars and starch, bioprocesses such as fermentation for vitamin production, biocatalysis in active pharmaceutical ingredient production, and other applications in textiles and leather, animal feed, pulp and paper, energy, metals, minerals and waste processing. McKinsey & Company [1] estimates that by 2010 the chemical industry could generate 10 to 20% of its sales revenue from chemicals derived from biotechnology. The largest component would be fine chemicals at 30 to 60% of sales from biotech, specialty chemicals (15%), polymers (6%), and bulk chemicals (12%). The most likely area of increased penetration

for biotechnology is in fine chemical/pharmaceutical manufacture, where it is estimated that \$30 billion to \$60 billion in additional value could be generated by 2010 as evident by the shift in demands toward biologics, chiral products, and natural products.

Increased petrochemical raw material cost (doubling of natural gas prices since 2000) and competition from low-cost producers such as China and India have eroded profit margins in the chemical industry. This has therefore encouraged ways to find the more cost-effective routes to existing and new chemical entities. Biotechnology capabilities are likely to be a technology differentiator for western companies as they face increased competition and commodization pressures. In polymers, biotechnology has been used in some applications, but it remains a largely niche area today. Biotech routes can be used in the production of certain monomers such as acrylamide, adipic acid, caprolactam, and dicarboxylic acids, and can be used in polymerization for making polyester. New polymers using biotech routes are DuPont's Sorona (1,3-propanediol), polylactic acid polymers (PLAs from Cargill Dow), polyhydroxyalkanoates (PHAs from Metabolix), and Nexia's Spider Silk. However, the key, but still elusive, target for white biotechnology is yet to penetrate bulk chemicals. Acetic acid, succinic acid, and some oleochemicals might be among the next bulk chemicals to become "biotech." The key to low-cost production here is the shift toward waste biomass as a feedstock for fermentation, which would substitute for petroleum-based feedstocks. Although biomass as a feedstock is potentially cost-competitive to petroleum-based feedstocks, there are still some challenges ahead. A switch to biomass would require building an entirely new value chain, with massive capital investments and collaboration among different players. Furthermore, technological advances such as in the enzymic conversion of cellulose are still needed for commercial applications [2].

Red biotechnology, on the other hand, has profoundly affected the pharmaceutical industry. Not only are there numerous new entrants that are developing new protein therapies, but the conventional, "small-molecule" drugs have also become more complex. The newer drugs are increasingly difficult to synthesize chemically and in many cases are chiral. Most regulatory authorities often require development of a single enantiomeric route. This poses huge synthetic challenges where the potential of biotech approaches for regioselective and enantioselective catalysis has become crucial. Furthermore, environmental implications in using undesired solvents and reagents in synthetic chemistry have prompted an increased use of biotech tools in this sector. Ideally, from an industrial viewpoint, it is desired to have a close integration between biotech and chemical catalysis at various stages of drug discovery and development to maximize its full potential. In the 1980s and 1990s several big pharmaceutical companies (Glaxo Wellcome, Merck, Schering-Plough, Pfizer, and Bristol-Myers Squibb) set up specialized biotech groups to complement internal R&D programs. Biotech enabled these giants to secure additional patent coverage and use it effectively as leverage for price negotiations with outsourcing partners.

The pharmaceutical industry is one of the biggest and most lucrative in the world, with annual sales of around \$400 billion. Pfizer rivals Microsoft in market capitalization, and the two are exceeded in size only by General Electric. Pfizer and other giants such as GlaxoSmith-Kline, Bristol Myer-Squibb, and Merck routinely report multibillion-dollar profits. But despite its outward strength, the industry is ailing. The "pipelines" of forthcoming drugs on which its future depends have been drying up for some time. In this competitive landscape the pharmaceutical sector is facing mounting pressures on number of fronts: (1) delivering new drugs to market rapidly and efficiently; (2) patents expirations and stiff competition from generic firms; (3) difficulty to maintain its market share and competitive edge; (4) the need to improve its product pipelines; (5) a gradual but steady shift of innovation base from big pharma to the biotech sector; (6) the realization that it is no longer cost-effective to do the entire R&D under a single roof; (7) tackling and understanding complex disease mechanisms; (8) navigating through complex regulatory hurdles; and (9) meeting shareholders' expectations. Although

globally research funding has doubled since 1991, the number of new drugs emerging each year has fallen by half. In 2003, America's Food and Drug Administration (FDA) approved only 21 "new chemical entities"—down from 53 in 1996. The more pharma spends on research, the less it seems to have to show for it. This is probably due to a combination of factors. One is the industry's obsession with producing "blockbusters" (drugs with annual sales of >\$1 billion). As the search for such bestsellers continues, firms may mistakenly be passing up smaller, but still profitable, opportunities, and as patents expire and revenues from old drugs dry up, costs are rising inexorably, not just in research but also in sales and marketing. So far, the industry has responded with cost-cutting, organizational changes, mergers, and acquisitions. The underlying challenge, however, is to address the *innovation deficit*.

There has been a technological explosion in the last decade. Rapid advances in technology have made significant impact on drug discovery. Achievements in human genomics, proteomics, and bioinformatics present huge possibilities to unravel new biological targets to understand disease states. The two much-hyped technologies, combinatorial chemistry and high-throughput screening (HTS), which appeared on the scene in the 1990s, promised to speed up the development of new drugs by exploiting automation, and generated lots of hits. It was hoped that testing many new compounds quickly would increase the rate at which new leads were produced. While these approaches looked promising, and the quantity improved, the quality did not. The number of new leads going into clinical testing did not increase, and enthusiasm for the new technologies waned. Similarly, the sequencing of the human genome was expected to revolutionize the process of drug discovery, and while it is undoubtedly a remarkable achievement, the link between genes and disease state is still obscure. Furthermore, the human genome project provided thousands of potential targets for new drugs that we must painfully sift through. This "information overload" has created a huge hurdle that industry must overcome. The genome is estimated to contain around 5000 pharmaceutically relevant genes; however, the 100 best-selling drugs target 43 genes between them, and the top 200, just 47—the whole industry is therefore, running on less than 50 genes. As a result, much effort is now being focused on using combinatorial chemistry and HTS more appropriately than in the past, and finding new ways to identify targets early, to determine the structure of proteins, and to test compounds for activity and behavior. The key aim is to distinguish winners from losers as early as possible. Failure can occur at any point in the discovery process, and the later the failure, the more costly the loss. A target may be important but not chemically tractable; drug compounds generated may not work, or not work well enough; a promising lead may turn out to be toxic—"fail early, fail cheap."

The enthusiasm for combinatorial chemistry also diverted attention away from compounds derived from natural products. Once a mainstay of pharmaceutical research, and the source of antibiotics and anticancer drugs, natural products were left behind in the rush to automation because they are complex and hard to make. But now they are making a comeback in the form of novel scaffolds for chemical libraries using combinatorial techniques. Despite the abundance of tools, most drug discovery and development efforts still fail because of a lack of understanding of how the drugs work, and an inability to predict reliably how the human body will handle them. To be successful the pharmaceutical industry must embrace and combine old and new techniques, to have the right blend of physiology, pharmacology, and target-oriented chemistry on one hand, and genomics, molecular modeling, and structural biology on the other. To be competitive pharma must also accelerate and improve efficiency in all aspects of drug discovery and development—the entire process typically costs \$900 million and takes 10 to 15 y (Figure 3.1). Only 1 in 1000 compounds tested makes it into human trials, and only 1 in 5 of those emerges as a drug. Outsourcing, strategic alliances, and partnership with the academic sector and biotech sector are now the key components of pharmaceutical R&D.

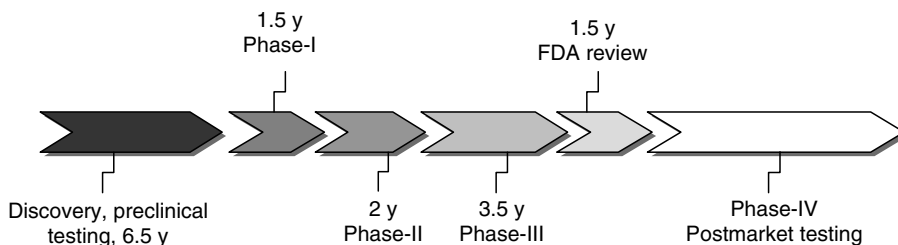


FIGURE 3.1 A typical drug discovery path in the pharmaceutical sector.

During 1989–2002 several major bioprocesses were developed at Glaxo Wellcome that were crucial to the market launch of many blockbusters including Epivir (anti-HIV), Lamivudine (anti-hepatitis), abacavir (Ziagen, anti-HIV), and Zanamavir (Relenza, anti-flu). In 2002, however, the biotech group at the newly merged GlaxoSmithKline was sadly disbanded. This chapter is a tribute to a decade of biocatalysis at Glaxo Wellcome and attempts to give a nostalgic but concise overview of some of the major bioprocesses developed in this era for the generation of antibiotics, antileukemic, anti-inflammatory, and antiviral agents, as well as several novel enabling technology platforms for production of optically pure amino acids and amines, and high-throughput experimentations.

3.2 β -LACTAM ANTIBIOTICS

The trinems **1** and **2**, bearing the tricyclic skeleton, are members of a novel class of totally synthetic β -lactam antibiotics discovered at Glaxo Wellcome, Italy (Figure 3.2). These are prepared by a multistep synthesis starting from the commercially available acetoxycetidinone **3** and the chiral alcohol **4** [3]. For large-scale production of **1** and **2**, an efficient synthesis of the chiral alcohol **4** was needed. We developed both enzymic and chemical resolution approaches for the preparation of multi-kg quantities of (+)-(1*S*, 2*S*)-*trans*-2-methoxycyclohexanol **4** from the corresponding racemate (\pm) *trans*-2-methoxycyclohexanol **5** [4]: firstly, a lipase-catalyzed transesterification of the unwanted (1*R*, 2*R*) enantiomer, followed by separation of the required alcohol **4** from the acylated species **6** by a simple partition method; and secondly, the synthesis of diastereomeric L-valine ester derivatives followed by separation of the required diastereomer **7** by fractional crystallization and hydrolysis back to the alcohol **4** (Figure 3.3).

For enzymic resolution, previous work had shown that the racemic acetate of *trans*-2-methoxycyclohexanol may be hydrolyzed to yield (–)-(1*R*, 2*R*)-2-methoxycyclohexanol in high enantiomeric excess (ee) using SAM-II lipase from *Pseudomonas* spp. [5], or pig liver acetone powder [6]. Similarly, the racemic butanoate of *trans*-2-methoxycyclohexanol was

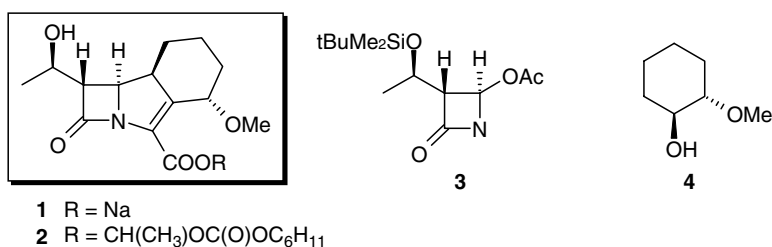


FIGURE 3.2 Structure of tricyclic β -lactam antibiotics and key chiral intermediates for their synthesis.

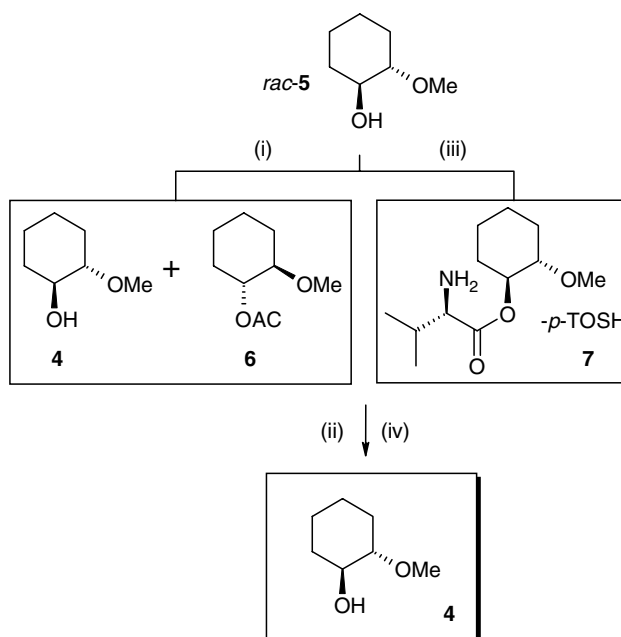


FIGURE 3.3 Chemoenzymic strategy for the resolution of racemic *trans*-2-methoxycyclohexanol **5**: (i) lipase, triethylamine, cyclohexane, vinyl acetate; (ii) aqueous extraction; (iii) L-valine, *p*-toluenesulfonic acid (*p*-TOSH), toluene, reflux, fractional crystallization; (iv) NaOH.

hydrolyzed by lipases from *Pseudomonas* and *Candida* spp. to yield the (–)-enantiomer of *trans*-2-methoxycyclohexanol [7]. Since we required the (+)-(1*S*, 2*S*) enantiomer **4**, we considered enantioselective acylation of the racemic alcohol **5** to provide a more direct route to our target compound without the need for subsequent ester hydrolysis. Amongst the lipases screened, enzymes from *Candida antarctica* and *Pseudomonas fluorescens* gave the highest ee for the enantioselective acylation of racemic **5**. Reactions were carried out in cyclohexane using vinyl acetate as acyl donor, and were monitored by chiral gas chromatography (GC) and high-performance liquid chromatography (HPLC). The immobilized *C. antarctica* lipase (CAL, Novozyme) exhibited excellent stability retaining over half of its initial activity after nine cycles of reuse. Process optimization showed the fastest bioconversions with cyclohexane as solvent. Acetaldehyde, resulting from transesterification of vinyl acetate, caused reaction inhibition, but this was reversible by simply washing the acetaldehyde-treated enzyme with cyclohexane, restoring its initial activity. Under optimized conditions CAL (37.4 g/L), vinyl acetate (1.7 M), racemic **5** (1.4 M) were used, with small quantities of triethylamine (0.16 M) to neutralize any acetic acid formed by hydrolysis of vinyl acetate; ee >98%, conversion 55% by GC. Cyclohexane was a particularly suitable solvent both for bioconversion and, due to its very low polarity, for the selective extraction of the required alcohol **4** into water at the end of a reaction cycle. The optically pure alcohol **4** was isolated in >99% ee (36% yield) by extraction into ethyl acetate followed by evaporation of organic solvent.

For L-valine resolution, we used chemical esterification of racemic **5** with L-valine in the presence of *p*-toluenesulfonic acid [8]. The formation of crystalline ester derivatives and the ability to optimize the efficiency of the resolution, either by varying the amino acid or by using alternative acids, made this approach very attractive to us for providing a large-scale resolution of the racemic *trans*-2-methoxycyclohexanol **5**. In practice, we found that a remarkably simple and efficient resolution could be achieved by heating the racemic alcohol and

L-valine in toluene at reflux in the presence of *p*-toluenesulfonic acid (1.3 molar equivalents). The desired (+)-L-valine ester **7** was obtained in good yield (typically >27%) and high diastereomeric excess (de 96% by proportionate mortality ratio, PMR) on filtration of the cooled reaction mixture. Treatment of the (+)-L-valine ester **7** in a mixture of *t*-butylmethyl ether and aqueous sodium hydroxide under phase-transfer conditions then gave the chiral alcohol **4** in the 95th percentile recovery.

In conclusion, whilst both chemical and enzymic methods were simple to operate and afforded material of good optical purity, the enzymic approach was advantageous, both economically and environmentally, and was the method of choice for production of the chiral alcohol **4** on a multi-kg scale.

3.3 ANTILEUKEMIC AGENTS

506U78 (2-amino-9- β -D-arabinofuranoyl-6-methoxy-9H-purine) was developed in the 1970s by Burroughs Wellcome as a potential antiviral agent but was dropped due to toxicity issues. After the merger with Glaxo, Glaxo Wellcome developed this compound for the treatment of leukemia [9,10]. 506U78 is a prodrug of *ara*-G (9- β -D-arabinofuranosyl guanine) (Figure 3.4). Given the difficulty in synthesizing *ara*-G using traditional chemical techniques, and its poor water solubility, we turned to 506U78, which is several times more soluble than *ara*-G and can be synthesized relatively easily using enzyme technology. *In vivo*, 506U78 is rapidly demethoxylated by adenosine deaminase to *ara*-G [11]; thus, many of the obstacles to using *ara*-G were circumvented by developing 506U78. Our colleagues at the former Burroughs Wellcome used uridine phosphorylase (UPase) and purine nucleoside phosphorylase (PNP), catalyzing net transfer of arabinose from a pyrimidine to a purine base with retention of the β -D configuration, for the synthesis of 506U78 (Figure 3.5). Each enzyme was cloned and overexpressed in independent *Escherichia coli* strains containing the corresponding genes on a multicopy plasmid.

In the UK, we developed, optimized, and scaled up a fermentation process for the enzyme production, and to simplify regulatory approval, all preparations of organism banks and fermentation processes were carried out in animal-free media: e.g., tryptone could be replaced with soya peptone without compromising cell yields and expression levels of each enzyme. Seed cultures (250 mL) of recombinant *E. coli* strains, for production UPase and PNP (Glaxo Wellcome collection), were grown at 37°C in a medium containing soya peptone (10 g/L),

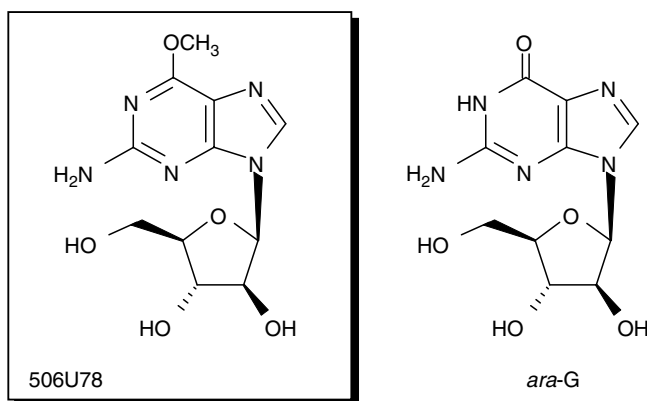


FIGURE 3.4 Structures of the antileukemic agent 506U78 and its prodrug *ara*-G.

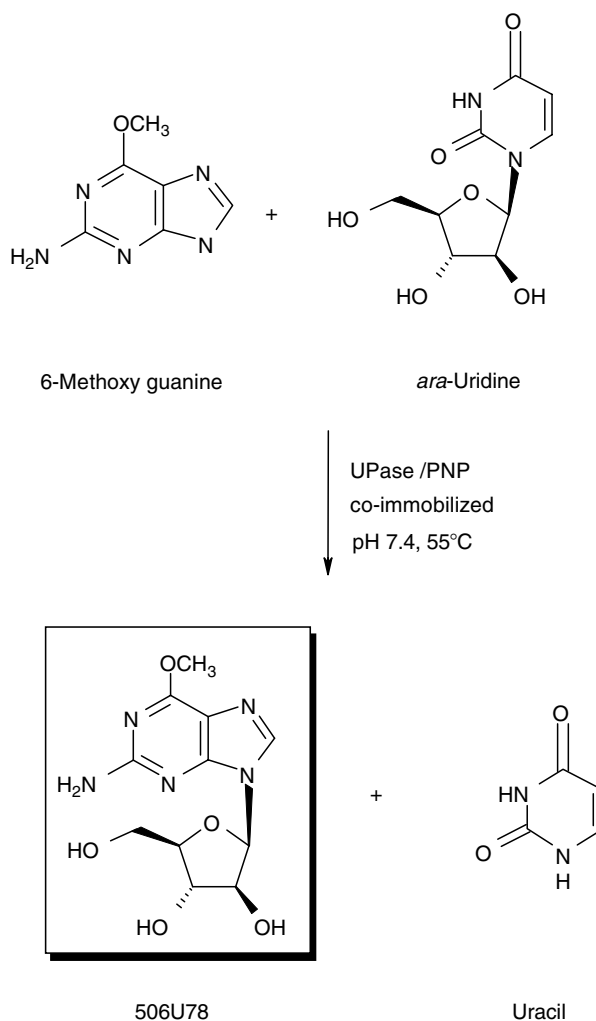


FIGURE 3.5 Enzyme-catalyzed formation of 506U78. UPase, uridine phosphorylase; PNP, purine nucleoside phosphorylase.

yeast extract (5 g/L), and NaCl (5 g/L). All media were supplemented with tetracycline (20 mg/L final concentration) for UPase, or kanamycin (50 mg/L) for PNP strains. Cultures were transferred to a production medium—soya peptone (10 g/L), yeast extract (5 g/L), potassium phosphate (13.1 g/L), sodium ammonium phosphate (3.5 g/L), citric acid (2 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/L), glycerol (6.3 g/L) postautoclave—when growth was in early to mid-log phase. Production stage flasks (2 L) were used to inoculate fermenters. Fermentation conditions were optimized to attain best enzyme production. Crude lysates of these enzymes were also found not to be stable at the high reaction temperature (55°C), but could be stabilized by direct co-immobilization of the enzymes onto an ion-exchange support (DEAE-52)—this also facilitated removal and recovery of the enzyme. Typically, bioconversions were carried out with up to 200 g/L substrate input and the co-immobilized enzymes could be reused several times in bioreactors. Upon completion of each cycle, beads were filtered and washed with the reaction buffer (10 mM potassium phosphate, pH 7.4). The crude product was crystallized from hot aqueous base (pH 11); this was followed by a final

purification step that involved product precipitation from hot water, after a charcoal treatment, to remove any potential endotoxin. This has formed the basis of a scalable process for production of 506U78 (Figure 3.5). The process was transferred to the factory to produce larger quantities for further evaluation.

To improve its pharmacokinetic profile, we found that esters of 506U78, in particular 5'-acetate, had a better water solubility and bioavailability [12]. All of the chemical synthetic approaches used, including selective acylation, or deacylation of the corresponding triacetate, showed poor selectivity, requiring chromatography to remove other undesired acetates, and were not amenable to scale-up [13]. We therefore embarked on a program to screen enzymes that would regioselectively acylate the 5'-hydroxyl position of 506U78, and optimized conditions for its production. Several enzymes (CAL, Novozyme; *Bacillus licheniformis* protease, Novozyme; *Mucor miehei* lipase, Novozyme; *B. licheniformis* protease, Altus; Savinase, Novozyme; *Alcaligenes* sp. lipase, Altus; Lipolase, Novozyme) were found to acylate 506U78 regioselectively but with up to 20% of the other related acylated products impurities. Novozyme-435, an immobilized preparation of CAL, was selected for process optimization—several parameters (solvents, acylating agents, temperature, substrate concentration) were systematically investigated and reactions were optimized to minimize the impurity levels (Figure 3.6). Preparative reactions were carried out under optimized conditions. Typically, bioconversions were carried out in anhydrous 1,4-dioxane, with vinyl acetate (20 to 50% v/v) as the acyl donor, and up to 100 g/L of 506U78. On completion of the reaction, the enzyme beads were removed by filtration and, after washing with neat methanol, were stored at 4°C before reuse. The immobilized enzyme was found to be stable when stored at 4°C and could be reused for another reaction cycle. The level of related impurities (3'-mono- and diacetates) was less than 0.5% and the reaction mixture could simply be evaporated to dryness to obtain the product in acceptable purity. This formed the basis of a scalable process for production of 5'-acetyl 506U78 (Figure 3.6) [14].

In summary, a co-immobilized UPase and PNP preparation from recombinant *E. coli* strains was used in the production of the antileukemic agent 506U78. Fermentation and bioconversion conditions were optimized and scaled up with up to 200 g/L of substrate input and the co-immobilized enzymes could be reused several times in bioreactors. In a parallel process an immobilized preparation of CAL was used to produce 5'-esters of 506U78, which were shown to have a better water solubility and bioavailability. Both processes were scaled up to produce much larger quantities for further evaluation.

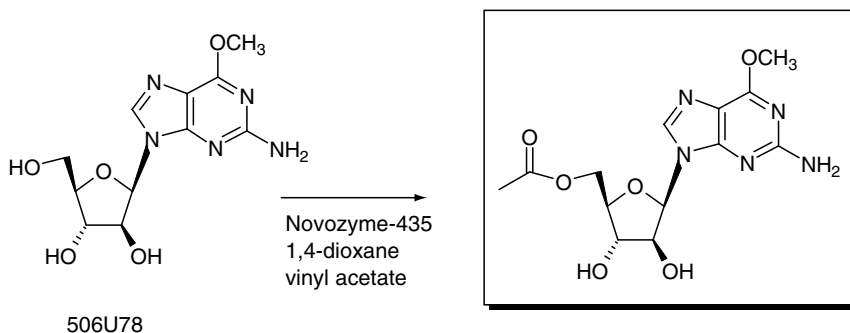


FIGURE 3.6 *Candida antarctica*-catalyzed acylation of 506U78. CAL, *C. antarctica* lipase: Novozyme-435.

3.4 ANTI-INFLAMMATORY AGENTS

In the early 1990s Glaxo Wellcome was developing a novel group of compounds with broad anti-inflammatory properties [15]. A key intermediate in the synthetic route is the nucleoside analog **9**, (1-[2-chloro-6-[(2,2-diphenylethyl)amino]-9H-purin-9-yl]-1-deoxy- β -D-ribofuranuronic acid) (Figure 3.7). Although chemical oxidation of the 5'-hydroxyl of precursor **8**, (2-chloro-*N*-(2,2-diphenylethyl)-adenosine), using transition metal oxidants (such as KMnO_4), was carried out in good chemical yields, this posed considerable problems for scale-up due to the heterogeneous nature of the reaction, and due to environmental and handling implications. The lack of regioselectivity of the oxidation reaction also necessitates the use of a protection-de-protection sequence to protect the 2'/3'-hydroxyl groups, thus introducing two extra steps into the route (Figure 3.7). We envisaged that an enzymic oxidation of a nucleoside precursor would offer potential for an improved synthesis—it is environmentally clean and obviates the need for protection of other functional groups.

Nucleoside oxidase is produced by *Pseudomonas* spp. and related Gram-negative bacteria [16–18]. Crude extracts of *Stenotrophomonas (Pseudomonas) maltophilia* exhibiting nucleoside oxidase activity were shown to convert the 5'-hydroxyl groups of natural purine and pyrimidine nucleosides to their corresponding carboxylic acids at analytical scale [19]—the purified enzyme catalyzes a two-step oxidation of a nucleoside by an aldehyde intermediate consuming one molecule of molecular oxygen [17,18]. Small-scale cultures of *S. maltophilia* (FERM BP-2252) were grown routinely at 25°C in 50 mL volumes of medium—yeast extract (25 g/L), glucose (30 g/L), K_2HPO_4 (1 g/L), KCl (1 g/L), MgSO_4 (0.5 g/L)—in shake flasks (250 rev/min, 5 cm throw). For larger-scale cultivations, 450 to 500 L fermentations were inoculated from two 8-h-old 5 L seed fermenters grown as above (2% v/v). Crude extracts of *S. maltophilia* were initially found to oxidize selectively the 5'-hydroxyl of **8** to generate **9** on mg scale; the substrate was also cleaved slowly to release the purine base, presumably by a

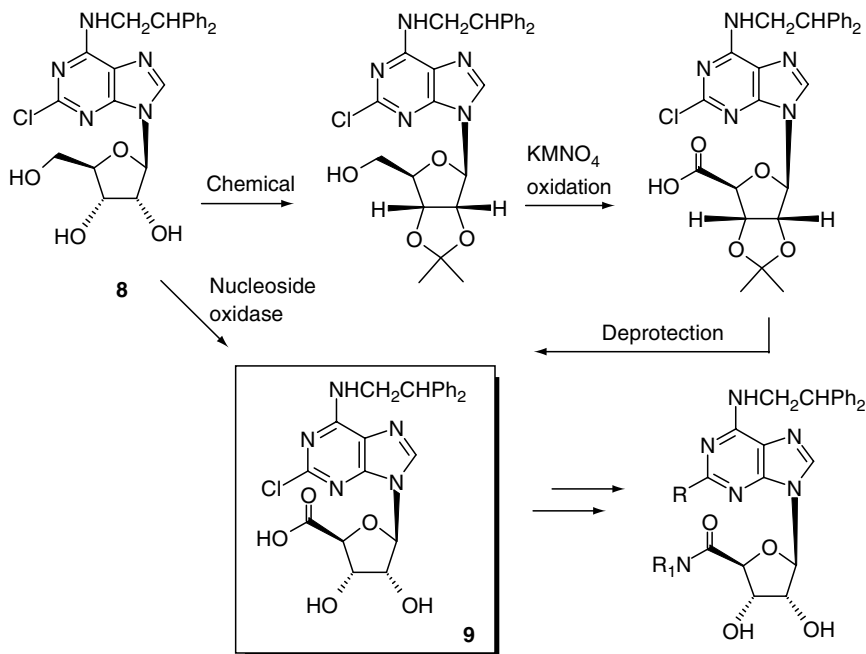


FIGURE 3.7 Nucleoside oxidase-catalyzed oxidation of **8**.

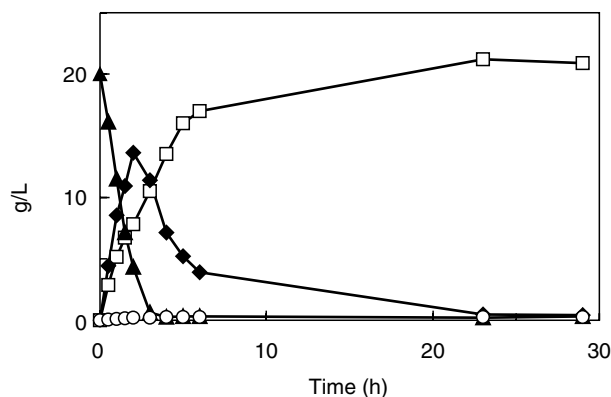


FIGURE 3.8 Production of **9** by crude extracts of *Stenotrophomonas maltophilia*. The reaction was carried out at room temperature in a magnetically stirred flask containing 3.5 mL of a clarified crude lysate (pH 6). **8** was added as a solid (70 mg) and stirred to obtain a homogeneous suspension. Periodically, samples were removed, diluted into the mobile phase, and the clarified solution assayed by HPLC. **8** (▲); **9** (□); aldehyde (◆); base (○).

phosphorylase activity [20] (Figure 3.8). This was, however, not considered to be significant when cells with a high nucleoside oxidase activity were used and crude extracts were found to be satisfactory for use in reactions without further purification. Crude extracts containing nucleoside oxidase were used in bioconversions with up to 20 g/L input with no evidence of inhibition at high substrate concentrations; **8** was quickly oxidized to **9** and reactions were completed within 24 h. Oxidation of **8** was shown to go through a transient formation of the corresponding aldehyde intermediate to produce the carboxylic acid in high chemical yields (Figure 3.8).

To simplify downstream processing, and to allow the enzyme to be reused, we chose to immobilize the enzyme directly from crude homogenates of *S. maltophilia* onto Eupergit-C beads (10 g dry beads/g protein). In free enzyme reactions, when the enzyme/substrate ratio was low, oxidation was not only slow but of limited duration and stopped before all the substrate was used; this was also evident with the immobilized enzyme. We found, however, that quinol has a dramatic effect on the oxidation of **8**; although initial reaction rates were significantly lower in the presence of 1 g/L of quinol, even at a substrate concentration of 20 g/L reactions continued up to completion in the presence of quinol, whereas the corresponding control reactions (without quinol) stopped at a product concentration of around 15 g/L (Figure 3.9). This was attributed to quinol having a protective role by stabilizing the enzyme during bioconversions. It was found that the same batch of enzymes could now be reused for at least five cycles and bioconversions were scaled up to produce larger quantities of **9** for further evaluation.

The nucleoside oxidase from *S. maltophilia* has been found to accept natural purine and pyrimidine nucleosides having ribose, deoxyribose, or arabinose as a sugar moiety, but does not oxidize the sugar in the absence of a base [16–18]. We envisaged using this enzyme to produce quantities (mg) of ribosides of unnatural purine bases and carbocyclic nucleosides for further testing. As the natural purine nucleosides (adenosine, guanosine, inosine, xanthosine) are all good substrates, it appears that the enzyme is quite tolerant of changes in the purine base, accepting either amino or carbonyl functionality at both positions 2 and 6. It was, however, surprising to find that **8**, with the bulky diphenylethylamino group at the 6-position, was also a good substrate. The enzyme is tolerant of different functionality at the 2-position including chloro **10** or phenyl ethyl amino **11** (Figure 3.10). Furthermore, the

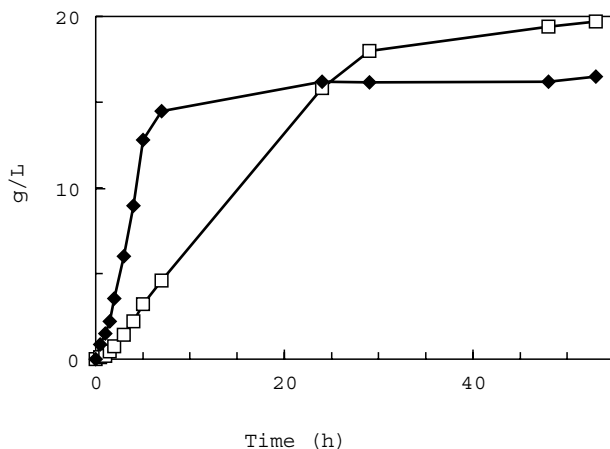


FIGURE 3.9 Reaction profile for oxidation of **8** using immobilized nucleoside oxidase from *Stenotrophomonas maltophilia* with quinol. The reaction (5 mL) was carried out in 50 mM potassium phosphate buffer (pH 6). **8** (100 mg) was added as a solid and stirred at room temperature to obtain a homogeneous suspension. The reaction was started by the addition of washed immobilized beads at 40% (w/v). At intervals, samples were removed, cleared of enzyme beads, and analyzed by HPLC. Reactions were carried out either in the presence (□) or absence (◆) of quinol at 1 g/L.

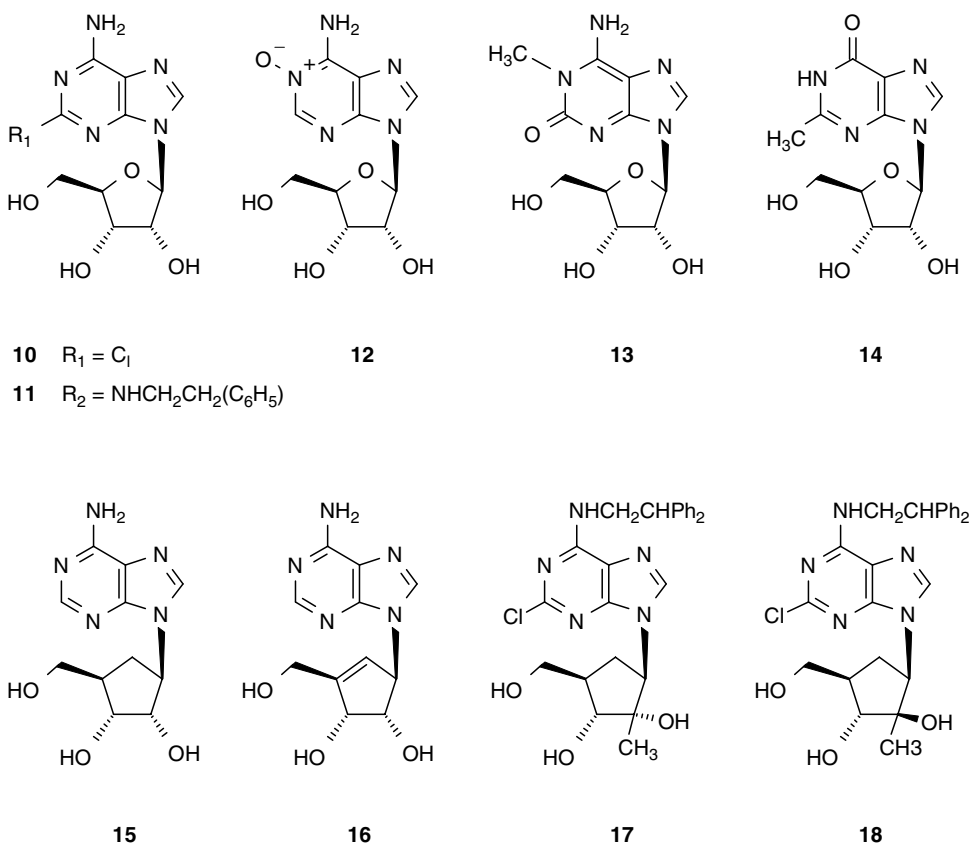


FIGURE 3.10 Substrate tolerance of the nucleoside oxidase from *Stenotrophomonas maltophilia*.

nitrogen at the 1-position can be modified to the *N*-oxide **12** or methylated in the case of methyl isoguanosine **13**. Interestingly, in the inosine series, with 2-methylinosine **14** as a substrate, the reaction tended to stop at the 5'-aldehyde intermediate and was not further oxidized. Carbocyclic nucleosides such as aristeromycin **15** and neplanocin-A **16** were also selectively oxidized to give the 5'-carboxylates (Figure 3.10). The enzyme did not, however, accept a methyl group at the 2'-position of the carbocyclic moiety **17–18** or the 2',3' acetonides of any of the natural nucleosides.

In summary, the synthetic utility of the nucleoside oxidase from *S. maltophilia* was exploited to produce the 5'-carboxylates of several purine nucleoside analogs, including the carbocyclic nucleosides aristeromycin and neplanocin-A on a preparative scale. This has formed the basis of a scalable process for the generation of nucleoside 5'-carboxylic acid derivatives. The enzyme has surprisingly wide substrate specificity toward unnatural nucleosides, especially in the base moiety [20].

3.5 ANTIVIRAL AGENTS

During the late 1980s and early 1990s a range of compounds were under evaluation at Glaxo Wellcome as potential antiviral agents. These were oxathiolane nucleoside Efavirenz (Lamivudine **19**), 2-aminopurine nucleoside Abacavir (Ziagen **20**), Zanamavir (Relenza **21**), carbocyclic nucleosides *c*-BVdU **22**, *c*-dG **23**, and Carbovir **24** (Figure 3.11). We went on to develop scalable biological routes, which were crucial to the market launch of several of these blockbusters (sales >\$1 billion), to complement our chemical synthesis programs.

3.5.1 EPIVIR (LAMIVUDINE)

(2'*R*-*cis*)-2'-deoxy-3-thiacytidine (3TC, Efavirenz, **19**) (Figure 3.11) has been approved by the FDA, and is marketed for the therapy of human immunodeficiency virus (HIV), in combination with Retrovir (Zidovudine, AZT) and Abacavir (Ziagen **20**), in adults and children. HIV is the causative agent of acquired immunodeficiency syndrome (AIDS), which is characterized by a chronic suppression of many immune functions and a concomitant increase in susceptibility to opportunistic infections [21,22]. Efavirenz is a potent and selective inhibitor of the reverse transcriptase enzyme, which is required for the replication of the HIV genome, catalyzing the conversion of the HIV RNA to a double-stranded DNA copy. Efavirenz is also active against hepatitis B virus (HBV) and is sold as Lamivudine in China. In the early 1990s when we began developing the racemic BCH189 (\pm **19**, Figure 3.12), licensed from Biochem Pharma, Canada, we found that in contrast to the majority of nucleoside analogs, which display antiviral activity primarily or exclusively residing in the "natural" β -D-isomer, the enantiomers of \pm **19** are equipotent *in vitro* against HIV-1 and HIV-2, but the "unnatural" β -L-(–)-**19** isomer (Efavirenz) (Figure 3.12) is substantially less cytotoxic than its corresponding "natural" β -D-(+)-isomer [23,24]. Clinical studies have shown Efavirenz to be well tolerated at high doses, with a good bioavailability and pharmacokinetic profile compared with other nucleoside analogs, and it does not appear to cause bone marrow toxicities *in vitro* [25,26].

Early supplies of the individual enantiomers for biological evaluation were made using preparative chiral HPLC; this method was, however, not amenable to scale up to provide the larger quantities of Efavirenz required for further evaluation. One of the initial routes considered was the enzymic resolution using 5'-nucleotidase and alkaline phosphatase that would allow access to both enantiomers of **19**. The chemically synthesized monophosphate derivative (\pm)-**27** was resolved using 5'-nucleotidase from *Crotalus atrox* venom, and the resulting

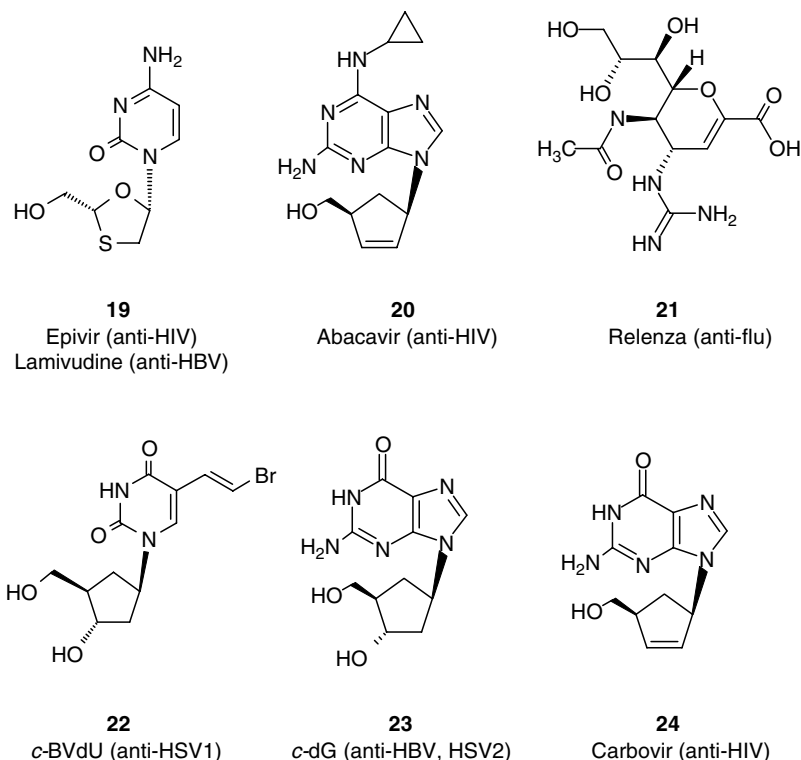


FIGURE 3.11 Selected structures of Glaxo Wellcome's antiviral agents.

mixture was separated by chromatography and purified on silica gel, to give (+)-**19** (ee > 99%, Figure 3.12). Hydrolysis of the remaining monophosphate (–)-**27** with alkaline phosphatase from *E. coli* (EC 3.1.3.1) afforded (–)-**19** (Epivir) in an optically pure form (Figure 3.12). To establish a chiral synthesis for Epivir, we needed to determine the absolute stereochemistry of the individual enantiomers of (+) and (–)-**19**. Epivir was treated with 4-bromobenzoyl chloride to afford the corresponding N⁴-amide; the presence of the heavy bromine atom allowed the absolute stereochemistry to be determined by x-ray crystallography, confirming the “unnatural” configuration of Epivir [27]. To produce much larger quantities of Epivir, the 5'-nucleotidase route was inefficient; the most expeditious way forward was to investigate a scalable end-stage resolution, by enantioselective deamination of (±)-**19** with cytidine deaminase [28] (EC 3.5.4.5), to support the development of Epivir (Figure 3.12). At that time, cytidine deaminase was not commercially available and had not been widely used for preparative transformations. The *E. coli* cytidine deaminase seemed a promising source as it was known to be inducible to reasonably high levels in enteric bacteria (*E. coli*, *Salmonella typhimurium*), allowing these organisms to grow rapidly with cytidine as sole nitrogen source [29]. Initially, small-scale reactions confirmed that (±)-**19** was a substrate and that the enantioselectivity was exquisite. The enzyme was partially purified by ammonium sulfate fractionation (55 to 75% saturation) from a clarified cell extract of a wild-type *E. coli* B. The enzyme (0.3 unit) was incubated with (±)-**19**, 0.33 mg in a 1 mL reaction volume, for 24 h; then, chiral HPLC analysis indicated that the (+)-**19** had been deaminated to give the uridine analog (+)-**25**, leaving Epivir, essentially optically pure [28] (Figure 3.12). We therefore chose to investigate *E. coli* as a source of large quantities of enzyme for use in production of Epivir, and developed a scalable route involving cloning and

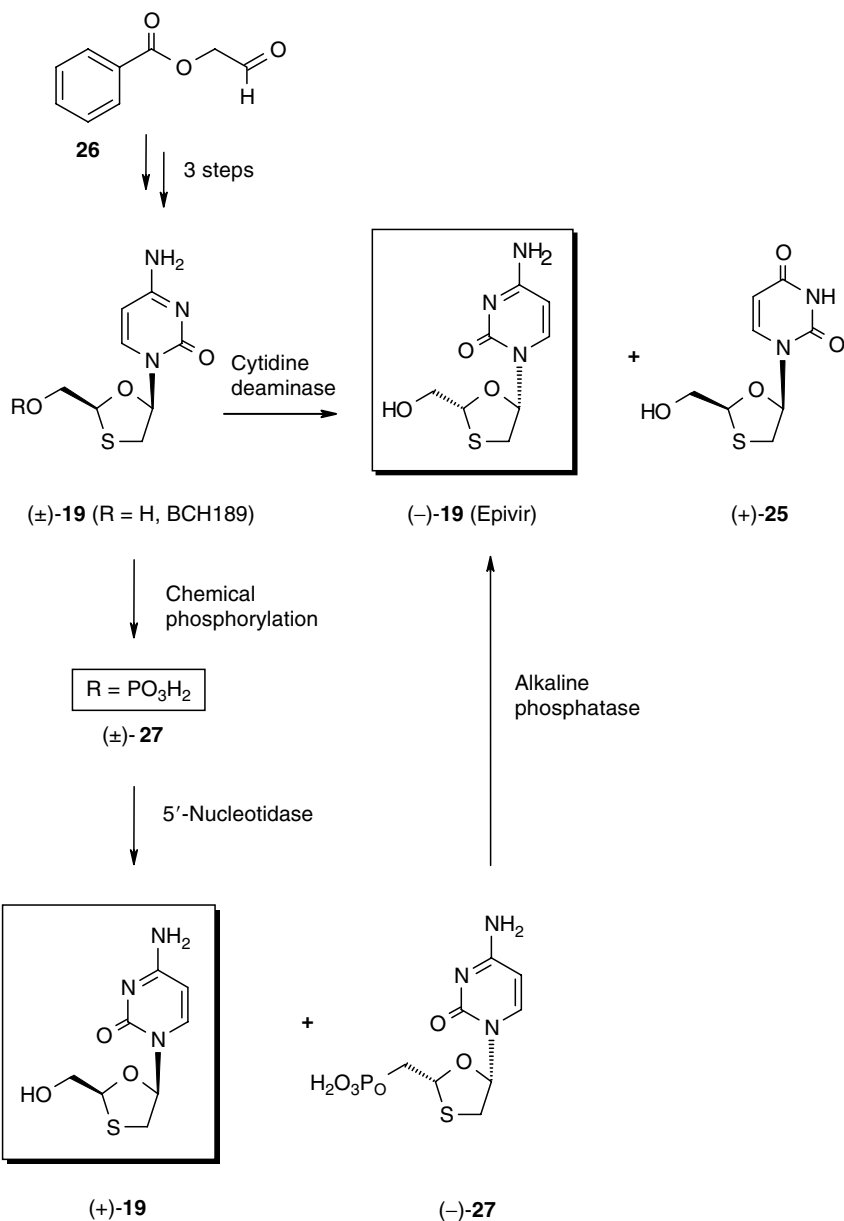


FIGURE 3.12 Synthesis of Epivir.

overexpression, fermentation and immobilization of cytidine deaminase for reuse, and development of an efficient isolation process that could be used in the factory to produce multi-tonne quantities of Epivir [28].

After the initial demonstration of activity in a clarified cell extract from *E. coli* B, we turned our attention to developing a robust procedure for isolation and immobilization of the enzyme. Cytidine deaminase was partially purified by column chromatography at a small scale; a 20-fold purification, with good recovery of the enzyme, was obtained by ion-exchange and hydrophobic interaction chromatography followed by ammonium sulfate precipitation. As the scale of operation was increased, the procedure was modified to include a batch

adsorption step rather than column chromatography; after cell disruption, the clarified lysate was adsorbed onto cellulose DE-52 and the enzyme was eluted with 0.3 to 0.5 M NaCl (50 to 70% recovery). The significant improvement in the production of cytidine deaminase with the constitutive mutant and especially the recombinant strain prompted us to investigate the possibility of simplifying enzyme preparation further by using crude extracts without enzyme purification. The batch adsorption step was, therefore, omitted from the isolation procedure. The cell extracts were found to be satisfactory for use in the biotransformation. There was no evidence of competing side reactions and the enzyme activity was found to be remarkably stable. Typically, 4 kg (wet weight) of *E. coli* 3732E cell paste was suspended in 20 L of lysis buffer—KH₂PO₄ (50 mM), EDTA (1 mM), DTT (1 mM), *p*-hydroxybenzoic acid ethyl ester (500 ppm), pH (7.5)—and disrupted by three passages through a Manton–Gaulin homogenizer. The extract was clarified using continuous centrifugation and/or microfiltration and concentrated approximately fivefold. The solution was further clarified by centrifugation (20,000g for 60 min) prior to immobilization. A 4-kg batch of the mutant strain (3732E) typically yielded 150,000 units of cytidine deaminase, whereas 15,000,000 units could be obtained from 4 kg of the clone (3804E); one unit of activity was defined as the amount of enzyme required to deaminate 1 μ mole cytidine/min at 25°C. For larger-scale operations, we found that even the clarification step could be omitted and the crude extract was directly immobilized onto Eupergit-C (Rhom Pharma, Darmstadt, Germany) without centrifugation, to give a stable enzyme preparation. At Glaxo's Greenford pilot plant in the United Kingdom, a 20-kg campaign was carried out as a series of 3-kg (500 L working volume) batches of racemic **19** (Figure 3.12) using immobilized cytidine deaminase from the constitutive mutant 3732E. Deamination of (\pm)-**19** was initially rapid, but approached completion through a very gentle asymptote. Careful chiral HPLC was needed to judge when the residual Epivir had reached sufficiently high optical purity (ee > 99.5%). In practice, cessation of alkalinization of the medium by ammonia release was a particularly useful indicator that the reaction was “completed.” The same batch of enzyme was used for at least 15 cycles. Although the reaction time increased from 35 h to more than 70 h, this was attributed primarily to physical loss of the beads during collection and washing. To improve volumetric productivity, the effect of increased substrate concentration was investigated; no inhibition was noted up to 30 g/L input. These higher concentrations were therefore used when the process was transferred to the factory [28].

Having successfully scaled up the bioconversion stage of the process, we focused on developing a simple and efficient strategy for isolation of Epivir. Initial work involved small-scale isolation procedures using ion-exchange chromatography (QAE Sephadex), followed by desalting of Epivir on a polystyrene divinyl benzene resin (XAD16) column and recovery by freeze drying. Neither the ion-exchange step, due to poor flow characteristics of the column, nor freeze drying were suitable for operation at larger scale. A series of resins were therefore tested and Duolite A113, a strongly basic polystyrene quaternary ammonium resin with a good capacity for the uridine analog [(+)-**25**, Figure 3.12], was selected and used for larger-scale operations. The freeze-drying step was also replaced by direct crystallization of product from the concentrated solution. At the completion of each cycle of reaction (500 L, 3-kg substrate input), the pH was adjusted to 10.5 with concentrated ammonia solution, and the mixture was applied to a column of Duolite A113 super resin in the OH[−] cycle. The uridine analog (+)-**25** adsorbed to the resin, whereas Epivir was not retained. The column was washed with 0.04% ammonia solution. This wash and the column spent, which contained the Epivir solution, were combined, the pH adjusted to 7.5 with concentrated H₂SO₄, and the mixture applied to an XAD16 resin column. The column was washed with distilled water and Epivir was eluted with 33% (v/v) acetone in water. Fractions containing Epivir were combined and concentrated approximately fourfold on a Balfour wiped film evaporator.

The concentrate was warmed to 50°C to dissolve any crystals and the solution was vacuum-filtered through Whatman-54 filter paper before further concentration to a slurry (3 L) using a rotary evaporator. After cooling, the crystalline product was recovered by filtration. Typically, 1.15 kg of highly pure Epivir (average recovery, 76%) was recovered from each 3-kg batch. Purity was better than 97% by HPLC and the ee was at least 99.8%. Using this approach, 20 kg of optically pure Epivir was isolated to support the initial development program. For further scale-up, the process was transferred to our production site at Ulverston, Cumbria, UK, where several tonnes of Epivir were produced, using immobilized cytidine deaminase from the recombinant strain.

One major drawback with the routes—(i) enzymic resolution of (\pm)-**19** using 5'-nucleotidase and alkaline phosphatase and (ii) enantioselective deamination of (\pm)-**19** with cytidine deaminase—discussed earlier is late-stage resolution of the racemic drug (Figure 3.12). In practice, 50% of the valuable starting material was unused and, as the scale of operation increased, alternative routes for the manufacture of Epivir were sought. Nevertheless, resolution using immobilized cytidine deaminase was the only scalable route *initially* available that enabled us to rapidly produce multitonne quantities of optically pure Epivir for clinical trials; this resulted in a 2-y clinical lead before a chemical asymmetric route was put in place.

As an alternative to end-stage resolution, we also investigated enzymic methods to effect resolution prior to the addition of the cytosine base. One such route considered was the enantioselective hydrolysis of racemic *trans*-5-propionyloxy-1,3-oxathiolane-2-methanol benzoate **28**, to obtain the key intermediate (–)-**28** [30] (Figure 3.13). The *trans*-oxathiolane benzoate **28**, synthesized from benzoyloxyacetaldehyde **26** (3 steps), was an attractive substrate because it could easily be accommodated into the existing synthetic route. Interestingly, **26** was also a common starting material for the synthesis of (\pm)-**19**, BCH189 (3 steps, Figure 3.12). A number of commercially available lipases and proteases were screened for the ability to hydrolyse racemic oxathiolane **28** enantioselectively. *M. miehei* lipase was identified as the most efficient biocatalyst. All other enzymes tested were less enantioselective than *M. miehei* lipase, but it is interesting to note that *C. cylindraceae* and *Chromobacterium viscosum* lipases and subtilisin showed the opposite enantioselectivity to *M. miehei* lipase, albeit in very low ee [5 to 15% ee (+)-2*S* isomer] [30]. Bioconversion of **28** with *M. miehei* lipase afforded enantiomerically enriched residual ester of the correct absolute stereochemistry, (–)-**2R**, for the subsequent synthesis of Epivir (2 steps, Figure 3.13).

In summary, our finding that *E. coli* cytidine deaminase can deaminate racemic **19** (BCH189, Figure 3.12) enantioselectively extends the understanding of the specificity of this enzyme, and it has become a generally useful reagent for resolution of unnatural cytidine nucleosides. The cloning and overexpression of the enzyme, under the control of a high-level inducible promotor, was essential to the development of a scalable process. Also crucial for developing a large-scale process was the demonstration that cytidine deaminase can be immobilized to give a stable enzyme preparation that can be reused many times in the reaction. The absence of substrate inhibition allowed high volumetric productivities to be achieved and assisted downstream processing. A robust scalable product isolation process was developed to yield crystalline Epivir. Here a simple two-column process, with adsorption–desorption rather than chromatographic steps, was used. Overall, yields through the resolution process of 76% were obtained. The overall process proved remarkably robust in a factory setting with batches of enzyme surviving entire production campaigns. The resolution with cytidine deaminase was the only scalable route available to us for synthesis of Epivir through much of the preclinical and clinical development of the drug. However, end-stage resolution was never likely to be the optimal economic route in the longer term, and chemical resolution of an early stage intermediate now forms the basis of the manufacturing process for Epivir [31].

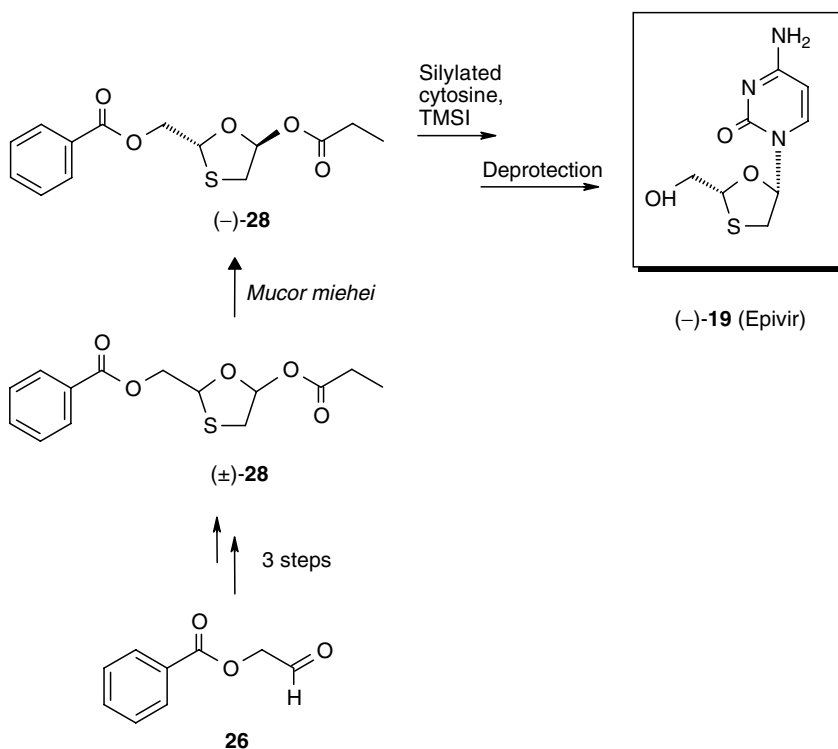


FIGURE 3.13 An alternative chemoenzymic route for synthesis of Epivir.

3.5.2 ABACAVIR (ZIAGEN)

Abacavir **20** (Ziagen) has been approved by the FDA for the treatment of HIV and HBV infections in adults and children—it is a selective and potent reverse transcriptase inhibitor [32] (Figure 3.14). This novel 2-aminopurine nucleoside analog is lipophilic and water-soluble, synergistic *in vivo* with protease and other reverse transcriptase inhibitors, as well as being well tolerated and orally absorbed with significant CNS penetration [33]. The triple combination regimens, involving the various reverse transcriptase and protease inhibitors, are often difficult to adhere to; patients may have to take between 10 to 20 pills/d at different times and, depending on the drugs involved, either with or without food and drink. In 2000 the FDA approved Trizivir, a combination therapy of a fixed dose of Abacavir (Ziagen), Retrovir (AZT), and Epivir (3TC)—the three nucleoside reverse transcriptase inhibitors (NRTIs) already approved by the FDA. Trizivir provides a well-tolerated and compact dosing regimen (one tablet twice a day, with no dietary restrictions), which can be particularly important in improving patient adherence. This new simple dosing regimen therefore addresses one of the most significant clinical challenges (patient adherence) faced in HIV treatment today.

The unsubstituted γ -lactam **31** (2-azabicyclo[2.2.1]hept-5-en-3-one) is a potential intermediate for the synthesis of Abacavir (Figure 3.14). The ChiroTech group (now part of Dow Chemical) in Cambridge, United Kingdom, had developed a process for the resolution of racemic **31** using γ -lactamase containing microorganisms such as *P. solanacearum* NCIMB 40249 and *Rhodococcus* sp. NCIMB 40213 [34,35]; these enzymes are, however, proprietary and not commercially available for general use. To circumvent ChiroTech's patent, we at Glaxo Wellcome envisaged that by activating the lactam ring with acyl-protecting groups

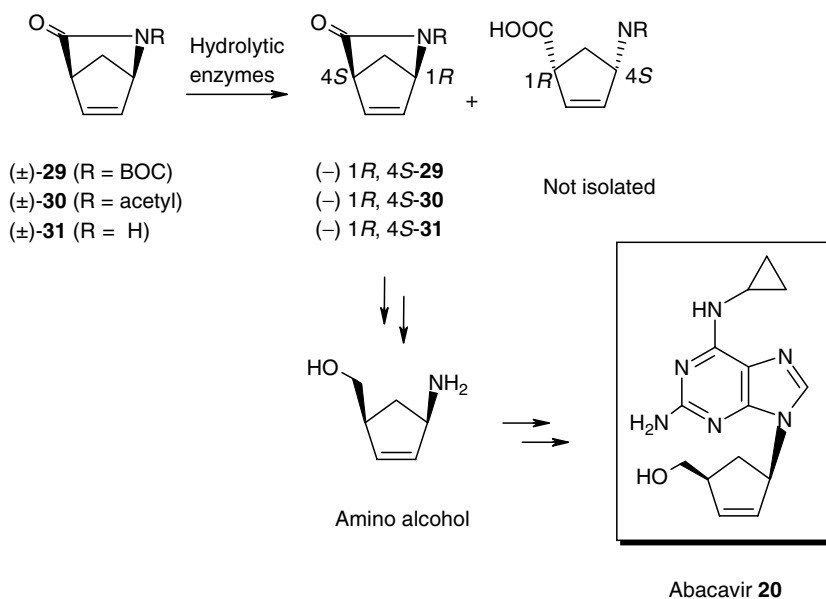


FIGURE 3.14 Production of Abacavir.

such as butyloxycarbonyl (BOC) or acetyl, we may be able to find a conventional hydrolytic enzyme, rather than needing a specialized γ -lactamase, that would hydrolyse the lactam bond of **29** and **30** enantioselectively. We therefore embarked on a program to produce the *N*-BOC-substituted γ -lactam **29** in an optically pure form for the synthesis of Abacavir [36].

A number of commercially available hydrolytic enzymes were screened for the ability to hydrolyse the lactam bond of racemic **29** [(±)-*tert* butyl 3-oxo-2-azabicyclo(2.2.1)hept-5-ene-2-carboxylate], enantioselectively (Figure 3.14). There was substantial chemical hydrolysis of the *N*-BOC protecting group under aqueous conditions, but this could be minimized if reactions contained up to 50% (v/v) of organic solvents such as tetrahydrofuran. We found surprisingly that several esterases, proteases, and lipases hydrolyzed (+) 1*S*, 4*R*-**29** to the corresponding *N*-acyl amino acid, leaving behind the residual (-) 1*R*, 4*S*-**29** of the correct absolute configuration for the synthesis of Abacavir (Figure 3.14). Savinase was most promising and reactions were found to be highly enantioselective. Savinase (subtilisin, EC 3.4.21.62) is a serine-type protease, manufactured by Novozyme, and is produced by submerged fermentation of recombinant alkalophilic *Bacillus* sp. It is cheaply available in bulk for use in detergent industry and is an active ingredient of washing powders [37]. Typically, reactions were carried out at 30°C in phosphate buffer (pH 8.0) containing up to 50% (v/v) organic solvent such as tetrahydrofuran and up to 100 g/L of racemic **29**. Reaction mixtures were monitored by reverse phase and chiral HPLC. Upon completion of reactions (50% conversion) the ee of (-)-**29** was better than 99%. The reaction mixture was filtered and (-)-**29** was isolated in good chemical yield (84% theoretical) by extraction into cyclohexane followed by evaporation of organic solvent [36]. Similarly, savinase hydrolyzed the lactam bond of racemic **30** (*cis*-2-acetyl-2-azabicyclo[2.2.1]hept-5-en-3-one) enantioselectively to afford (-) 1*R*, 4*S*-**30** ee > 99% (Figure 3.14); interestingly, savinase did not hydrolyse the unactivated racemic **31**. This was the first example of the use of detergent enzymes for the large-scale production of pharmaceutically important active intermediates. This has formed the basis for the development of a simple and scalable process for preparation of optically pure *N*-substituted γ -lactams for the production of Abacavir [36,38].

In summary, we have developed a simple, efficient, cost-effective, and practical enzymic procedure for the preparation of *N*-substituted γ -lactams, using a readily bulk-available commercial protease, in very high optical (ee > 99%) and chemical yields (84% theoretical). This process was scaled up and integrated into the chemical route, which resulted in a significant overall cost saving to manufacture Abacavir. We subsequently used our strong patent coverage for price negotiations with outsourcing partners [38].

3.5.3 ZANAMAVIR (RELENZA)

Relenza **21** (2,3-didehydro-2,4-dideoxy-4-guanidinyl-*N*-acetylneuraminic acid, Figure 3.15) is a potent and selective inhibitor of influenza virus sialidase (neuraminidase) and has been approved by the FDA for the treatment of types A and B influenza—the two types most responsible for flu epidemics. The neuraminidase removes sialic acids from glycoconjugates aiding virus penetration into respiratory tract and releases mature viroins from the infected cells. Relenza works by preventing the spread of influenza from one cell to another within the respiratory tract. To target the site of infection directly, Relenza is inhaled (using a Diskhaler device) into the airways, where the flu virus replicates, and begins to function to destroy the

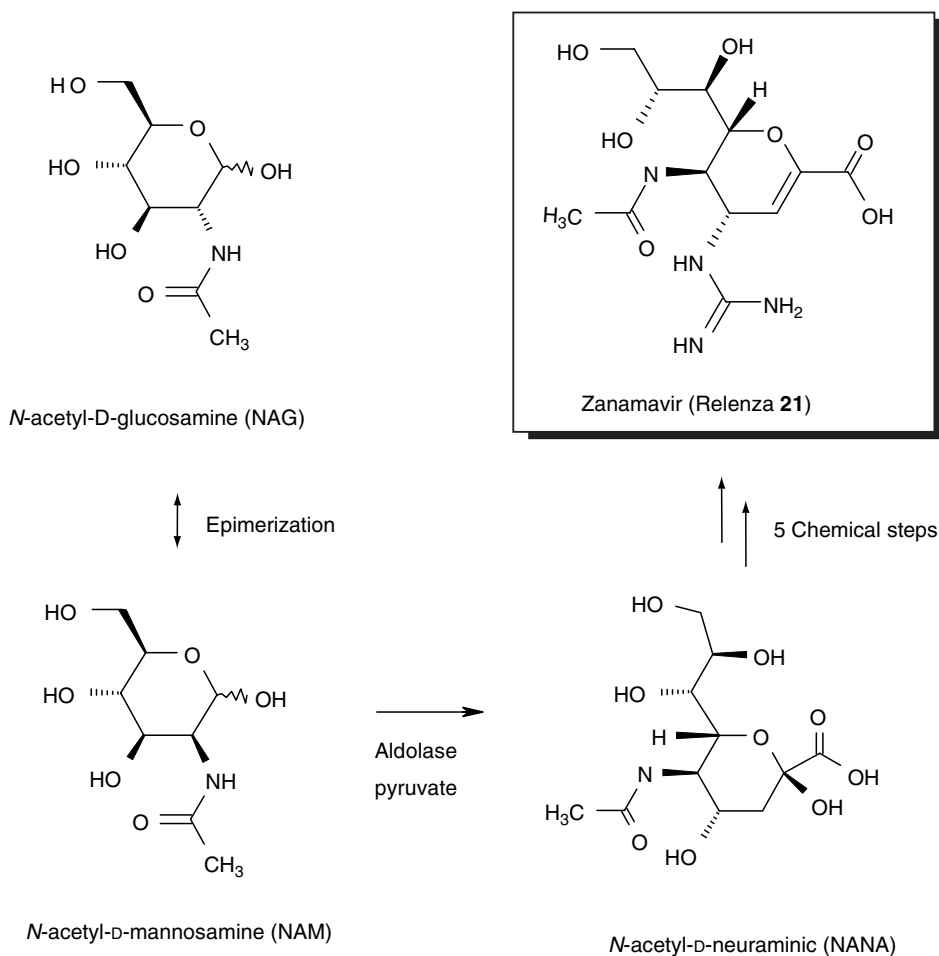


FIGURE 3.15 Chemoenzymic production of Relenza.

virus. Relenza was licensed from Biota Australia after a collaborative effort that led to its rational design from the x-ray crystal structure of sialic acid bound to influenza sialidase with the aid of molecular modeling and computational chemistry techniques [39].

N-acetyl-D-neuraminic acid (NANA, Figure 3.15) is the key intermediate for synthesis of Relenza. NANA is the most prominent of the sialic acids, a group of 9-carbon amino sugars incorporated at the terminal positions of glycoproteins and glycolipids, which play an important role in a variety of biological recognition processes [40]. The chemical synthesis of NANA is lengthy, requiring complex protection and deprotection steps, and does not offer much potential for economic large-scale production [41]. NANA can be isolated from biological materials such as milk, eggs, edible birds' nests [40,42], or bacterial cell wall polymer colominic acid [43–45], but levels are modest and purification difficult. The most promising option for long-term supply appeared to be the use of the NANA aldolase enzyme. Several bacteria contain this enzyme, which appears to have a role in the catabolism of NANA that is cleaved to pyruvate and *N*-acetyl-D-mannosamine (NAM). Under appropriate conditions, the enzyme can be used in reverse to synthesize NANA (Figure 3.15).

There are, however, a number of hurdles to be overcome for the development of an efficient biochemical process for production of NANA: (i) NAM is expensive and must be produced *in situ* by epimerization of *N*-acetyl-D-glucosamine (NAG) at C₂ [46], but the equilibrium lies in favour of NAG; (ii) the K_m for NAM is high (0.7 M) [47]; (iii) NAG is inhibitory [47]; and (iv) pyruvate is usually used in excess (10 molar excess) to push the equilibrium over to NANA, which necessitates the removal of large amounts of residual pyruvate, but pyruvate and NANA are difficult to separate. The synthesis of NANA using the aldolase enzyme either from *E. coli* or *Clostridium perfringens* had been previously reported by several groups [48–50]. These groups developed batch processes for the production of NANA from NAM and pyruvate using free or immobilized NANA aldolase. In order to drive the equilibrium toward NANA, the cheaper pyruvate was generally used in large excess, making the downstream processing rather difficult.

The Wandrey group at Juelich developed an elegant continuous process for NANA synthesis introducing the NANA-2-epimerase enzyme for epimerization of NAG and integrating the epimerization with NANA synthesis in an enzyme-membrane reactor [47]. Although this process has an excellent space time yield, the epimerase enzyme is not freely available—the product stream is very dilute and gradient chromatography is required for purification, making scale-up difficult. The Marukin Company [51] also achieved “simultaneous” epimerization and aldol condensation by operating the enzyme process at high pH (e.g., pH 10.5), but this led to significant compromises of both enzyme activity and epimerization rate, and again the product isolation issue was not adequately addressed. None of the reported procedures, however, offered a fully effective and integrated process solution for the large-scale production of NANA using NANA aldolase, and in particular methods for isolation of NANA from the reaction mixture were inadequate. We therefore embarked on the development of two approaches for NANA production that surmounted most of these problems [52,53].

It was clear that any approach to NANA synthesis would require a plentiful source of enzyme. We cloned the enzyme from *E. coli* and overexpressed it in an inducible system (*tac*-promoter) [53]. As very high expression levels were achieved (10 units/mg protein, representing 30% of cell protein), we hoped that we would not need to purify the enzyme further. Indeed, reactions with crude extracts were clean, with no observed side reactions. To minimize the processing required we simply homogenized the bacterial cells and directly immobilized the enzyme from crude extracts onto Eupergit-C beads without any clarification. Cell debris did not interfere with immobilization of NANA aldolase and could be washed away after the immobilization step. The immobilized enzyme was stable for at least ten batch reaction cycles.

Our initial strategy for production of NANA was to carry out the aldolase reaction on equilibrium mixtures of NAG and NAM (4:1 ratio), resulting from base-catalyzed epimerization of NAG, using a 5 to 7 molar excess of pyruvate to drive the equilibrium toward the NANA product (Figure 3.15). This resulted in more than 90% of NAM being converted into NANA; at completion, the reaction thus comprised NANA (21 g/L), NAM (2g/L), NAG (65 g/L), and pyruvate (56 g/L). To isolate NANA we removed pyruvate initially by complexation with bisulphite rather than resorting to the (low-capacity) ion-exchange separation of NANA and pyruvate. Indeed, after removing sodium ions with an IR120(H⁺) column, pyruvate was complexed very effectively using an A113 column in the HSO₃⁻ form. The column could be regenerated by simply using hot water to strip pyruvate and recharging with sodium metabisulphite—in principle the pyruvate could be reutilized. As the only remaining acid, NANA was selectively bound onto an anion-exchange resin, Duolite A113 PLUS(OAc⁻), eluted and crystallized by addition of acetonitrile. To improve the volumetric productivity for NANA, we studied the effect of increasing NAG/NAM concentration in equilibrium mixtures (4:1 ratio of NAG/NAM) and found out that high levels of NAG are grossly inhibitory to the aldolase reaction. To avoid the inhibition, we therefore investigated the possibility of enriching NAG/NAM mixtures for NAM.

In the second approach, a selective precipitation of NAG using isopropanol was developed to produce an NAM-enriched mixture (1:5 ratio of NAG/NAM). This was used in the reaction at a very high NAM concentration (up to 20% w/v) so that NAM itself drives the reaction and it is only necessary to add a small molar excess of pyruvate (e.g., 1.5-fold) (Figure 3.16). At the “conclusion” of the reaction, the mixture comprised NANA (155 g/L), NAM (13 g/L), NAG (24 g/L), and pyruvate (29 g/L) (Figure 3.17). Under these conditions, NANA could now be crystallized directly from the reaction mixture simply by the addition of acetic acid. Furthermore, we found that upon completion of the reaction, after filtering off

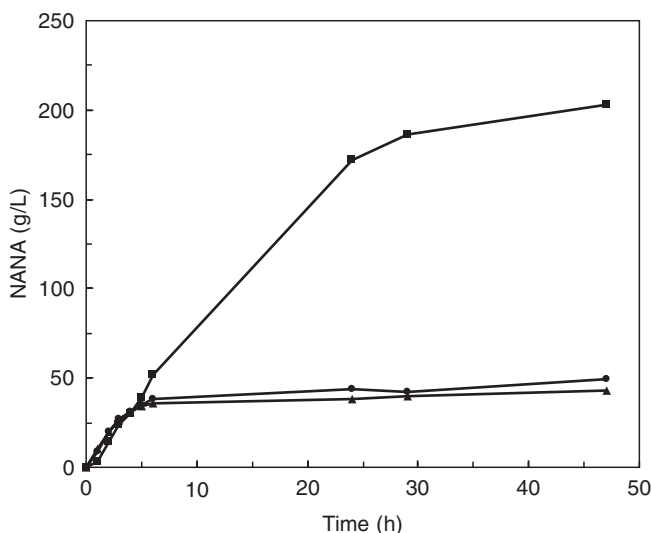


FIGURE 3.16 Comparison of production of NANA from NAM-enriched and equilibrium mixtures. Reactions (20 mL) were carried out at 20°C using either NAM-enriched (■) or equilibrium mixtures (●, ▲). The molar ratio of pyruvate/NAM was either 2:1 (■, ▲) or 5:1 (●). Reactions were started by the addition of 8 g wet weight of washed immobilized enzyme beads. The NAG/NAM concentrations were as follows: 66:224 g/L (■), 169:43 g/L (●, ▲).

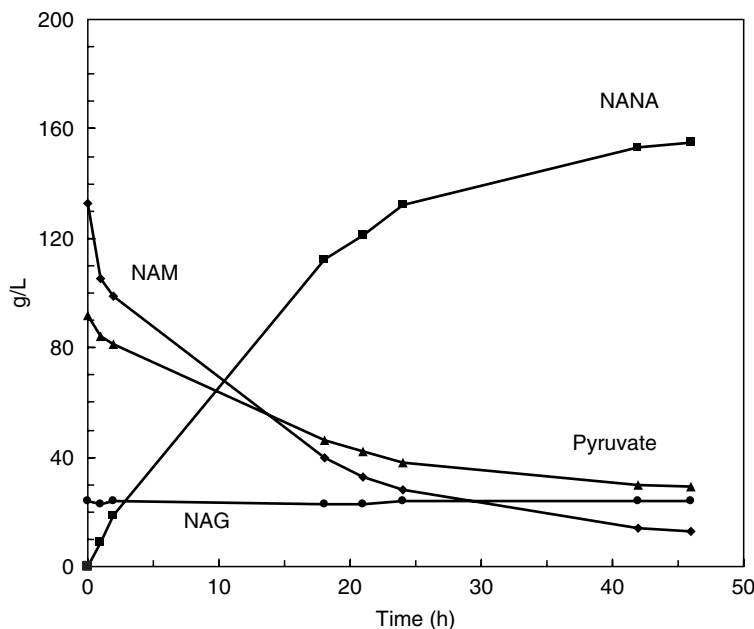


FIGURE 3.17 Production of NANA from an NAM-enriched mixture. The reaction was carried out at 20°C in a 7 L LH-fermentor containing 3.5 L of an NAM-enriched mixture. Sodium pyruvate (442 g) was added in a 1.5 molar ratio with respect to NAM. After adjusting the pH to 7.5 with NaOH, reaction was started by addition of 1.7-kg wet weight of washed immobilized enzyme beads. NANA (■), pyruvate (▲), NAG (●), NAM (◆).

the immobilized enzyme beads and concentrating the reaction mixture, we could achieve a NANA concentration of approximately 200 g/L by adding six volumes of acetic acid to give crystalline NANA in excellent purity [53].

In order to understand the contribution to sialidase binding made by each of the groups on the dihydropyran ring of Relenza, we used the aldolase technology to produce multi-quantities of various NANA analogs, for structure activity relationship (SAR) studies. In one such study, using 2-deoxy-glucose as substrate in the aldolase reaction, **32** was produced and used to synthesize **33** and **34** in a one-pot process that lacked a substituent at the 5-position of the dihydropyran ring [54] (Figure 3.18). Compounds **33** and **34** were evaluated as inhibitors of influenza A and B sialidase and were shown to display markedly reduced affinity for the viral enzymes when compared to Relenza [55]. Subsequent evaluation of these compounds against influenza virus sialidase has established the critical importance of the 5-acetamido group for good binding affinity of Relenza.

In summary, we developed two processes for NANA production, which have both been operated at substantial scale [52,53]. In the first process, the use of a large molar excess of pyruvate (five- to sevenfold) to drive the equilibrium to NANA has been made feasible by development of a bisulphite complexation method for pyruvate removal from the reaction mixture. In the second process, the development of a method for enrichment of NAM in NAG/NAM mixtures has allowed NAM to be used at very high concentration, and this obviates the need to use a large molar excess of pyruvate. The NAG residue recovered from the enrichment procedure can of course be recycled through the epimerization procedure. Under these circumstances it has been possible to develop a method for recovery of NANA from the reaction mixture by a simple crystallization: [NANA] > 150 g/L, overall

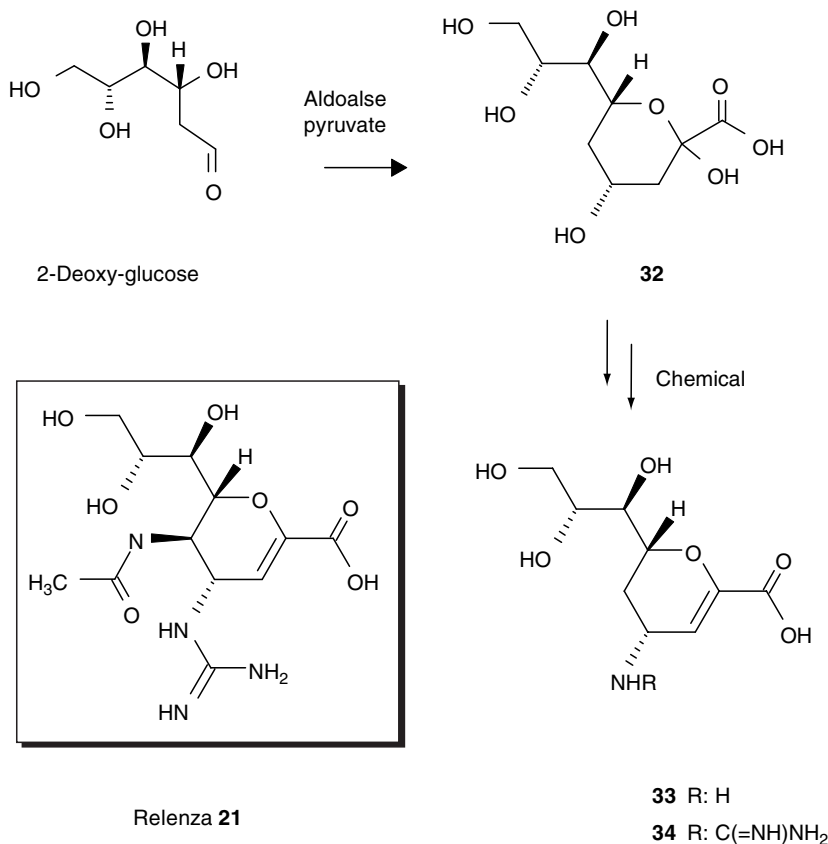


FIGURE 3.18 Aldolase-catalyzed bioconversion of 2-deoxy-glucose.

process yield 75% from NAM, purity of crystalline NANA >99%. In the pilot plant multi-kg quantities of NANA were produced using a recycled stable immobilized aldolase preparation. In the factory setting the same batch of enzymes was reused for more than 2000 cycles in batch column reactors, without any significant loss of activity, to produce multitonne quantities of NANA. This was the first example of an integrated process for industrial scale application of NANA aldolase, which was crucial for the market launch of Relenza.

3.5.4 CARBOCYCLIC NUCLEOSIDES

We at Glaxo Wellcome had spent a number of years investigating the potential of carbocyclic nucleosides as antiviral agents. Carbocyclic analogs of purine and pyrimidine nucleosides have anti-HIV and antiherpetic properties [56–58]; traditionally, however, these compounds have been synthesized and tested initially in racemic form. Where the enantiomers had been examined, biological activity shown by the racemate was found to reside primarily in the “natural” enantiomer, whereas the corresponding “unnatural” isomer was found to be inactive or showed substantially reduced potency [58–61]. Enzymes have frequently been used to resolve the enantiomers of carbocyclic and 2',3'-dideoxynucleoside analogs for further biological testing [62–66]. For example, adenosine deaminase was used to prepare optical isomers of a wide range of carbocyclic analogs of purine nucleosides [31,59,64,65], whereas

other carbocyclic nucleosides were resolved by enantiospecific hydrolysis of their monophosphates [31,58].

We used two basic approaches to introduce chirality in the synthesis of carbocyclic nucleosides *c*-BVdU **22**, *c*-dG **23**, and Carbovir **24** (Figure 3.11): (i) resolution of intermediates prior to base addition; and (ii) synthesis from the natural antibiotic aristeromycin **15** and neplanocin-A **16** (Figure 3.10) produced by the filamentous bacterium *Streptomyces citricolor*.

3.5.4.1 Resolution of Intermediates to Carbocyclic Nucleosides *c*-BVdU and *c*-dG

(+)-Carbocyclic 2'-deoxy-5-[(*E*)-2-bromovinyl]uridine **22** (*c*-BVdU) and (+)-carbocyclic 2'-deoxyguanosine **23** (*c*-dG) (Figure 3.11) are potent antiviral agents; (+)-**22** possesses activity against herpes simplex virus 1 (HSV-1) and varicella zoster virus (chicken pox and shingles) *in vitro* and *in vivo*, while (+)-**23** is active against HSV-2, human cytomegalovirus, and hepatitis B virus [67,68]. As an alternative to total asymmetric synthesis of **22** and **23**, and to avoid late-stage resolution, we envisaged the introduction of an enzymic or microbial step, to obtain key homochiral synthons, at an early stage in the synthetic strategy. One approach considered was the enzymic resolution of ester intermediates, prior to the addition of the base, that could be used in the synthesis of **22** [62]. We initially followed the work of Sicsic et al. [69] who had used pig liver esterase (PLE) for the resolution of 4-*cis*-acetamido-cyclopent-2-enecarboxylic acid (\pm)-**35** (Figure 3.19). We were disappointed, however, to find that the optical purity of the products (1*R*,4*S* residual ester, ee 28%) was very modest and had been overstated (ee 87%) in the paper due to an error in the optical rotation value for the homochiral ester. The acid product (1*S*,4*R*) rather than the residual ester (1*R*,4*S*) was of the correct absolute stereochemistry for synthesis of nucleoside analogs (Figure 3.19). We decided, therefore, to look at the benzoyl amino compound (\pm)-**36**, which was resolved more effectively using PLE and to our surprise with the correct opposite enantioselectivity (Figure 3.19). This highlights that the effect of substitution remote from the center of hydrolysis can profoundly affect the enantioselectivity of the enzymic hydrolysis of 4-amino-cyclopentane-carboxylic acid derivatives [62]. Although the enantioselectivity was still relatively modest, the

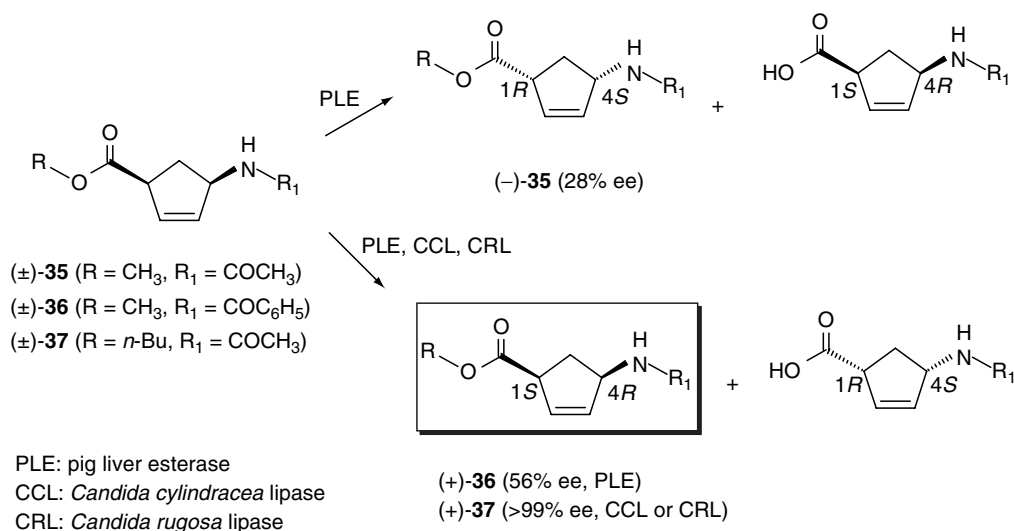


FIGURE 3.19 Enzymic resolution of carbocyclic nucleoside precursors.

residual ester product [(+)-1*S*,4*R* **36**, ee 56%] was of the correct absolute stereochemistry for synthesis (8 steps) of (+)-*c*-BVdU **22** (Figure 3.20). We were nevertheless able to improve the enantioselectivity by careful attention to the reaction conditions, particularly the cosolvent used; for example, methanol at 5% (v/v) increased the enantioselectivity of reactions by at least twofold. Interestingly with dimethyl sulphoxide (DMSO) at 50% (v/v) the enantioselectivity was reversed such that the residual ester was enriched in the wrong (–)-1*R*,4*S* enantiomer [62]. Although the enantioselectivity was not complete, the residual ester product could be obtained in good optical purity by allowing the reaction to proceed beyond 50%; for example, the residual ester could be obtained in 90% ee (65% yield) by allowing the reaction to proceed to 65% conversion or 95% ee (58% yield) at 69% conversion [62]. We were also interested to note that *C. cylindracea* or *C. rugosa* lipases were reported some years later to resolve higher ester of the acetamido compound *n*-butyl (±)-**37** (Figure 3.19) or *n*-hexyl esters; the residual esters (+)-1*S*,4*R* were obtained in excellent optical purity (ee > 99%) [70].

In an attempt to shorten synthetic routes to **22** and **23**, we also investigated an alternative method using the stereoselective reduction of **38** by growing cells of *M. circinelloides* [71]. Optimization of fermentation conditions, pH, and temperature resulted in the production of the desired intermediate (+)-**38**, in an optically pure form (ee > 98%; yield, 62%), which was used for the convergent syntheses of (+)-**22** (3 steps) and (+)-**23** (4 steps) (Figure 3.20).

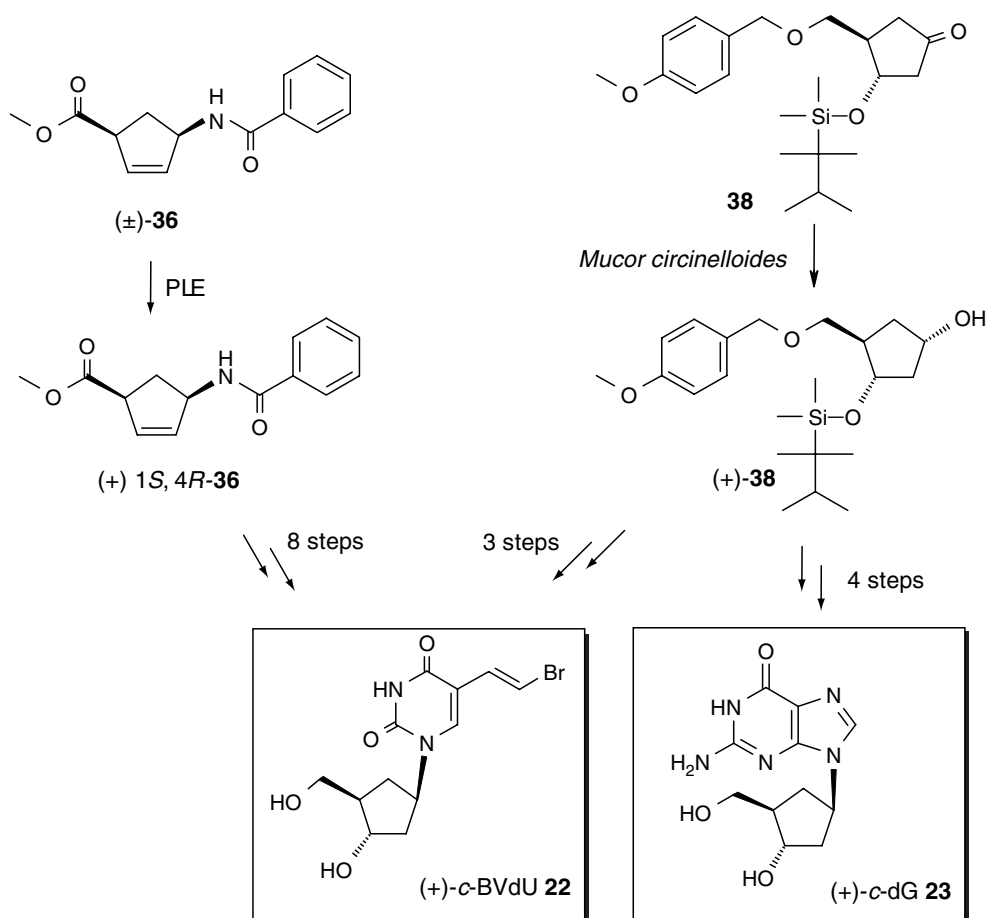


FIGURE 3.20 Chemoenzymic synthesis of (+)-*c*-BVdU **22** and (+)-*c*-dG **23**.

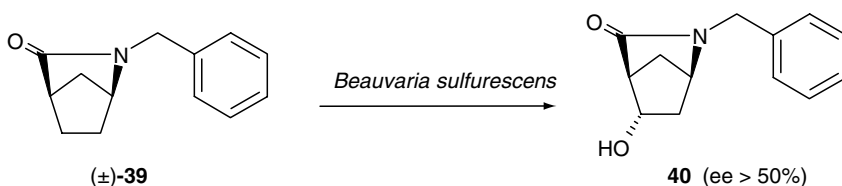


FIGURE 3.21 Microbial hydroxylation of the bicyclic lactam **39** as a potential intermediate for carbocyclic nucleoside synthesis.

Furthermore, the α -hydroxy (+)-**38** has been shown to be a versatile intermediate that can be used for the convergent synthesis of a variety of chiral purine and pyrimidine carbocyclic nucleoside analogs [71].

There was potential for a still more attractive route by the enantioselective, regiospecific, and stereospecific hydroxylation of the bicyclic lactam (\pm)-**39** (Figure 3.21). Although the 5-*exo*-hydroxylation of (\pm)-**39** was reported previously using *Beauveria sulfurescens*, the optical purity of the product **40** was not measured quantitatively [72]. Although we obtained similar results, we were disappointed to find that the desired hydroxylated product had an ee of only 11%. Screening of other fungi increased this beyond 50%, but yields were quite modest and the benzyl lactam proved to be intractable to further chemistry [73] (Figure 3.21).

3.5.4.2 Aristeromycin and Carbovir (c-d4G)

The 2',3'-didehydro-2',3'-dideoxycarbocyclic nucleoside, (\pm)-Carbovir, is a potent and selective inhibitor of HIV *in vitro* [74]. Its hydrolytic stability and ability to inhibit infection and replication of the virus in human T-cell lines at concentrations 200- to 400-fold below toxic levels has made Carbovir a potentially useful antiretroviral agent. (–)-Carbovir **24** (Figure 3.11) is approximately twofold more active than the corresponding racemate, whereas the (+)-enantiomer is at least 75-fold less active than (–)-**24** [75]. One of the routes to (–)-**24** considered at Glaxo Wellcome was synthesis from the chiral natural product (–)-aristeromycin **15**. The potential of aristeromycin as a chiral pool starting material for synthesis of carbocyclic nucleosides was recognized at Glaxo Wellcome in the mid-1980s and we invested a substantial effort in the development of fermentation and isolation conditions for this antibiotic. (–)-**15** was a very attractive starting material because it was readily available as a secondary metabolite of *S. citricolor* and would afford (–)-**24** without the need to resort to an optical resolution. Synthesis of (–)-**24** from (–)-**15** involved nine steps requiring two distinct transformations: (i) an adenine to guanine base interconversion; and (ii) introduction of the 2',3'-double bond from the 2',3'-diol [76]. One approach for the base conversion involved the hydrolytic deamination of *cis*-4-[2,6-diamino-9*H*-purin-9-yl]-2-cyclopentene-methanol dihydrochloride (–)-**41** using adenosine deaminase; (–)-**41** was prepared from aristeromycin in eight steps [76] (Figure 3.22). To obtain sufficient material for further biological evaluation, the chemoenzymic route was investigated and a process developed to produce (–)-Carbovir **24** on a kg scale [77]. We used commercial preparations of adenosine deaminase (calf intestinal mucosa and *Aspergillus* sp.), and following standard purification techniques directly adsorbed the enzyme onto an anion-exchanger to provide a rapid method for its recovery (50 to 340 units/mg protein). Bioconversions with the partially purified enzyme were carried out at pH 7.5 in fermenters (up to 70 L working volume). At 20 g/L input of (–)-**41**, the product (–)-Carbovir **24** (Figure 3.22) was much less soluble, crystallizing during the course of reaction and was subsequently recovered by filtration (>90% theoretical yield). To allow enzyme reuse, adenosine deaminase was immobilized onto Eupergit-C to give

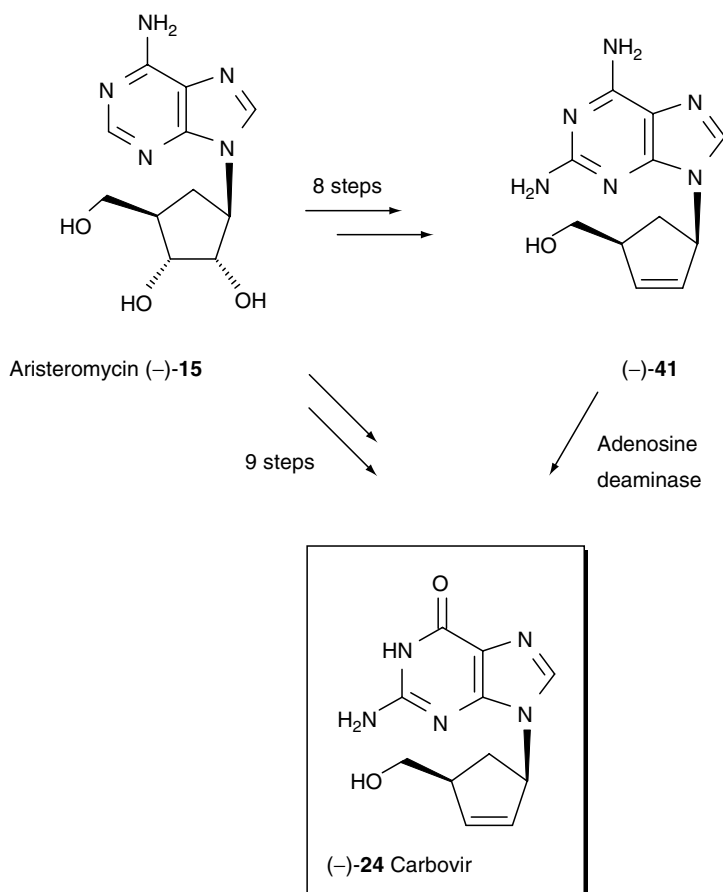


FIGURE 3.22 Chemoenzymic synthesis of Carbovir (-)-24 from aristeromycin.

a stable enzyme preparation. Given the low solubility of (-)-Carbovir, the concentration of (-)-41 was reduced to 2.5 g/L in bioconversions with immobilized enzyme so that the beads could easily be recovered from the reaction mixture without interference from the product. The enzyme was reused up to 10 cycles without any significant loss of activity. This work demonstrated the potential of adenosine deaminase as a catalyst for large-scale production of optically pure (-)-Carbovir **24** [31]. In contrast to Abacavir (-)-20, however, Carbovir suffers from poor oral absorption, limited brain penetration, low aqueous solubility, and potential for renal and cardiac failure [31]. Despite the structural similarity between Abacavir and Carbovir (Figure 3.11), it is remarkable that an apparently small change in the molecule can have such a profound effect on its biological activity.

Another approach to base interconversion arose through an observation during isolation of biosynthetic mutants of the aristeromycin production strain. Neplanocin-A **16** (Figure 3.10) was fed to block mutants looking for conversion of aristeromycin; one mutant was found not to produce aristeromycin, but to modify the base moiety of the added neplanocin-A, especially by deamination. On feeding aristeromycin, this was converted to carbocyclic inosine, carbocyclic xanthosine, and carbocyclic guanosine (Figure 3.23). The conversion of aristeromycin to carbocyclic inosine by *Streptomyces* sp. from the “mutant” culture was rapid and the ability to do this was widespread amongst the organisms we investigated. Further conversion of carbocyclic xanthosine or carbocyclic guanosine was much slower. Carbocyclic

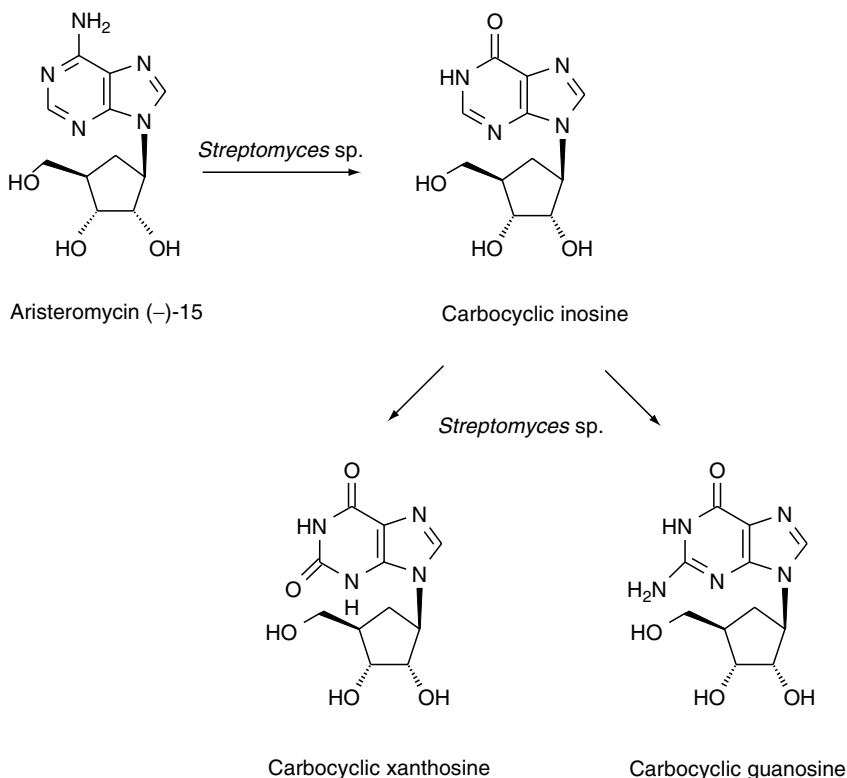


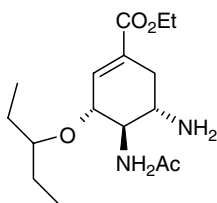
FIGURE 3.23 Microbial base interconversion of adenine to guanine.

inosine could be added to the cultures and was transformed to carbocyclic xanthosine and/or carbocyclic guanosine, but neither of these was further converted. Conditions could be manipulated to favor either carbocyclic xanthosine or carbocyclic guanosine as final products [73] (Figure 3.23).

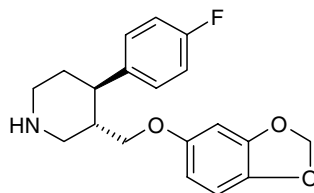
3.6 ENABLING TECHNOLOGIES

3.6.1 CHIRAL AMINES

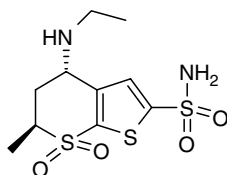
Chiral amines are valuable synthons that feature prominently in many agrochemicals and pharmaceutical drug pipelines. A recent survey of the top 400 drugs revealed the widespread prevalence of compounds containing amino groups attached to asymmetric centers [78]. The combined 2002 sales of only 12 blockbusters containing chiral amines was in excess of \$10 billion, covering a variety of indications including depression, bacterial infection, atherosclerosis, prostatic hypertrophy, growth failure, migraine, and Alzheimer's [78] (Figure 3.24). Chiral amines have great utilities as catalysts for asymmetric synthesis [79], resolving agents [80], and chiral auxiliaries/chiral bases [81]. Methods for the preparation of chiral amines are largely based on resolution of racemates (limited to 50% yield), either by recrystallization of diastereomeric salts [82–83] or by kinetic resolutions of racemates using hydrolases [84–88]. To improve reaction yields beyond 50%, asymmetric approaches have been developed using (i) transaminases for the conversion of ketones into chiral amines [89–92]; (ii) microbial reduction of oximes into chiral amines [93]; and (iii) asymmetric hydrogenation of imines



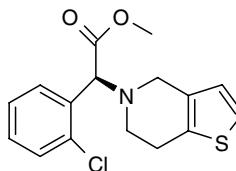
Roche — Tamiflu (anti-flu)
Sales > \$1 billion



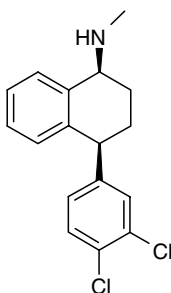
GSK — Paxil (asthma, depression)
Sales ca. \$3 billion



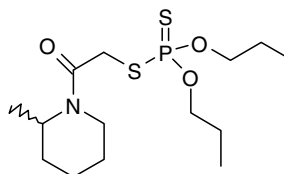
Merck — Trusopt (elevated
intraocular pressure)
Sales \$455 million



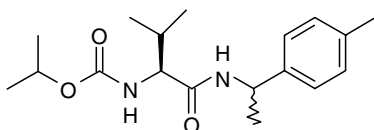
Sanofi, BMS — Plavix (atherosclerosis)
Sales \$3 billion



Pfizer — Zoloft (depression)
Sales \$2.8 billion



Syngenta — Piperophos (herbicide)
Sales \$24 million



BayerAG — Iprovalicarb (fungicide)
Sales > \$20 million

FIGURE 3.24 Structures of selected agrochemicals and pharmaceutical drugs containing chiral amino groups.

using chiral ruthenium catalysts [94–96]. Alternative strategies are based on dynamic kinetic resolution (DKR) of amines that employ enzymes in combination with transition-metal catalysts for *in situ* racemizations [97–100]. For example, in the DKR approach, Reetz and Schimossek [98] developed a chemoenzymic route for the synthesis of α -methylbenzylamine derivatives using CAL in combination with a palladium (Pd/C) catalyst to racemize the

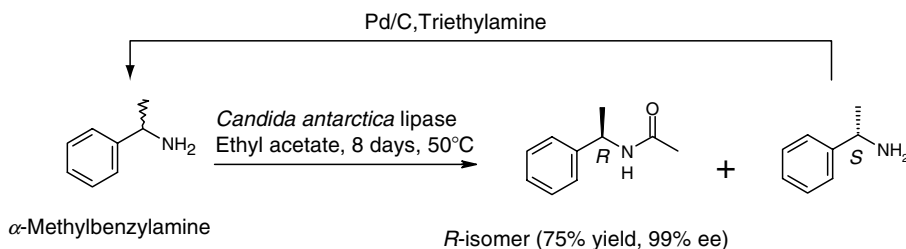


FIGURE 3.25 Dynamic kinetic resolution using palladium for *in situ* racemization.

undesired *S*-amine isomer (Figure 3.25). Although yields greater than 50% were obtained (ee > 99%, *R*-amide), there were compatibility issues between the enzymic and the chemical step due to the harsh conditions required for racemizations. This, together with the very slow racemizations rates (up to 8 days), has unfortunately limited the general utility of this approach. Current commercial processes for production chiral amines include:

1. BASF's ChiPros technology [97] based on lipase-catalyzed resolutions; this is, however, a multistep process requiring acylation, deprotection, and subsequent large-scale separation of the amine product.
2. Cambrex, using Celgene's transaminase technology [89,90], can be operated either in "resolution" or "synthesis" mode with yields >50%; however, the *R/S*-transaminases are not commercially available, the substrate range is rather limited to primary amines, and a very limited range of amino acceptors can be used in the resolution mode (Figure 3.26).
3. Biokatalyse, using a laborious multistep route to produce α -methylbenzylamine [101] (ee > 99%, 87% yield) (Figure 3.27).

In the 1990s we were interested to develop biocatalytic routes to chiral amines and amino acids to complement our chemical development programs. In collaboration with Universities

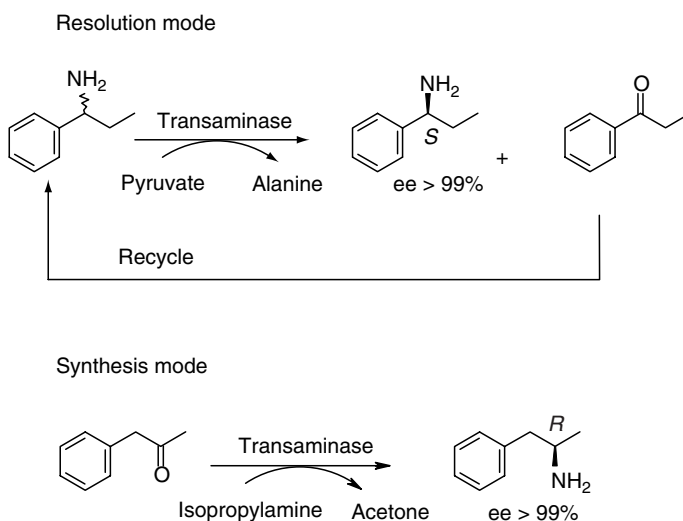


FIGURE 3.26 Celgene's (Cambrex) transaminase technology for production of chiral amines.

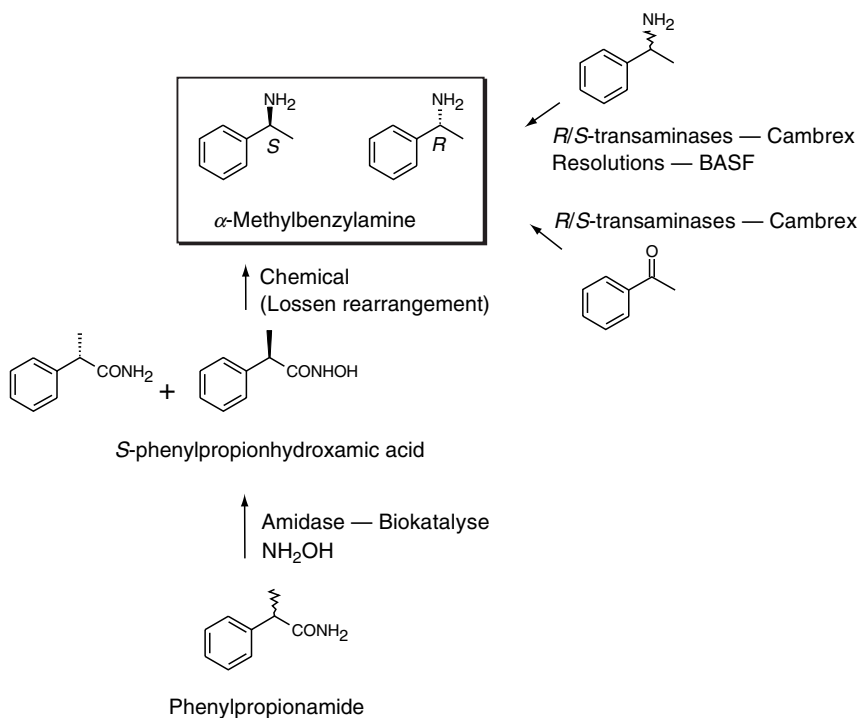


FIGURE 3.27 Commercial production of chiral amines.

of Warwick and Edinburgh, we at Glaxo Wellcome developed two elegant processes: (i) resolution of amines by “substrate engineering” [102]; and (ii) deracemization of amino acids and amines, in high optical and chemical yields, using enantioselective oxidases coupled with nonselective chemical reducing agents [103–105]. After the merger with SmithKline Beecham, however, our deracemization technology was “transferred” to Ingenza (a start-up company from Edinburgh University), who now exploits this approach for the synthesis of chiral amines and amino acids.

3.6.1.1 Resolution of Amines by Substrate Engineering

As a general rule of thumb, kinetics resolutions of esters of chiral acids **42** are more likely to succeed using esterases (e.g., porcine liver esterase) than lipases, whereas the resolution of esters of chiral alcohols **43** are more likely to succeed using lipases than esterase (Figure 3.28). This “functionality reversal,” therefore, greatly extends the use of lipases in resolution processes, and is an innovative concept of considerable practical potential [102]. Only a

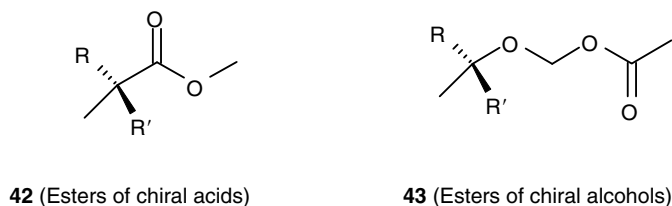


FIGURE 3.28 Enzymic resolutions by “functionality reversal.”

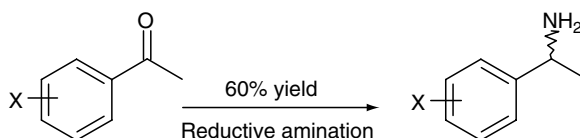
limited number of esterases are available commercially—these tend to be mainly from mammalian sources, which further limit their large-scale applicability. In contrast, however, numerous lipases are now available from microbial sources (e.g., *P. fluorescence* and CAL) in bulk quantities. In collaboration with Professor Crout at Warwick University, United Kingdom, we therefore envisaged a process for the production of either *R*- or *S*-isomers of chiral amines by “functionality reversal” using readily available lipases [102].

In this approach, we argued that functionalizing the amino group, with an ester linkage as close as possible to the chiral center, might make it more amenable to lipase resolutions. To access both isomers, the amine function should be regenerated from both the residual substrate and the acid product. Such an approach can be regarded as “substrate engineering,” complementary to “protein engineering,” which is used to tailor the enzyme to match a particular substrate. Several racemic-substituted 1-phenylethylamines (α -methylbenzylamine) were synthesized from the corresponding readily available acetophenones. These were then used in the synthesis of ethyl and octyl oxalamic esters **44a–g** in good chemical yields (Figure 3.29). Initially the ethyl oxalamic ester of 1-phenylethylamine **44a** was screened against a panel of lipases; CAL B (CAL, Novozyme-435) showed the highest enantioselectivity (*E* 11) for the hydrolysis of the *R*-oxalamic ester (Figure 3.29). Both the residual substrate and the corresponding acid product could now be readily hydrolyzed chemically to generate either the *R*- or *S*-chiral amine (Figure 3.29). Further optimization through modification of the ester chain length (C_2 to C_8) revealed that the enantioselectivity was significantly improved with the extension of the alkyl chain; the enantiomeric ratio *E* increased from 11 to 104 with the octyl group (Figure 3.30). Bioconversions with octyl oxalamic esters **44b–g** proceeded with very high degree of enantioselectivities (*E* 30 to 100); the *R*-oxalamic acids and the *S*-oxalamic esters were conveniently hydrolyzed chemically to the corresponding optically pure amines (Figure 3.29). Interestingly, in contrast to CAL, lipases from porcine pancreas (PPL) and *P. fluorescence* (PFL) showed opposite *S*-selectivity hydrolyzing the *S*-oxalamic esters (Figure 3.30). Furthermore, in sharp contrast to CAL, increasing the ester chain length from methyl to octyl had very little effect on the enantiomeric ratio with PPL and PFL (Figure 3.30). We also analyzed the selectivity of CAL and the influence of increasing ester chain length using published x-ray crystal structure of CAL [106]. Using computational modeling (minimization and dynamics programs), with mapping and calculations of the likely binding sites for both enantiomers with varying chain length, we found a likely “methyl pocket” in the active site of CAL that preferentially accommodates the methyl group of *R*-oxalamic ester of 1-phenylbenzyl amine resulting in *R*-oxalamic acid product. The active site seems to be narrow and U-shaped, allowing entry of the benzyl group—there is also a hydrophobic binding region that is involved in the binding of the ester chain up to octyl length, and any further increase beyond C_8 does not appear to have any significant effect on enantioselectivity of CAL [107].

Current approaches for commercial production of chiral amines are limited only to primary amines (Figure 3.27). We therefore wondered if our process could be used to produce chiral secondary amines. We were surprised to find that under nonoptimized conditions the octyl oxalamic ester **45** was kinetically resolved with moderate selectivity (*E* 4) by CAL giving *R*-oxalamic acid and *S*-oxalamic ester, which, after chemical hydrolysis, afforded both isomers of *N*- α -dimethylbenzylamine (Figure 3.31). Similarly modest selectivity (*E* 3) was observed with octyl oxalamic ester **46** resulting in the *R*- and *S*-isomers of 5-(3-pyridyl)-2-pentylamine (Figure 3.31). Given the apparent structural similarities between **44b** (*E* > 100) and **45** (*E* 4), this suggests that the additional methyl group might interfere with binding at the methyl pocket in the active site [107].

In summary, we have developed a novel and practical method for the resolution of a range of readily synthesized amines by “substrate engineering.” This simple process is capable of

Synthesis of racemic amines



Synthesis and resolution of oxalamic acid esters

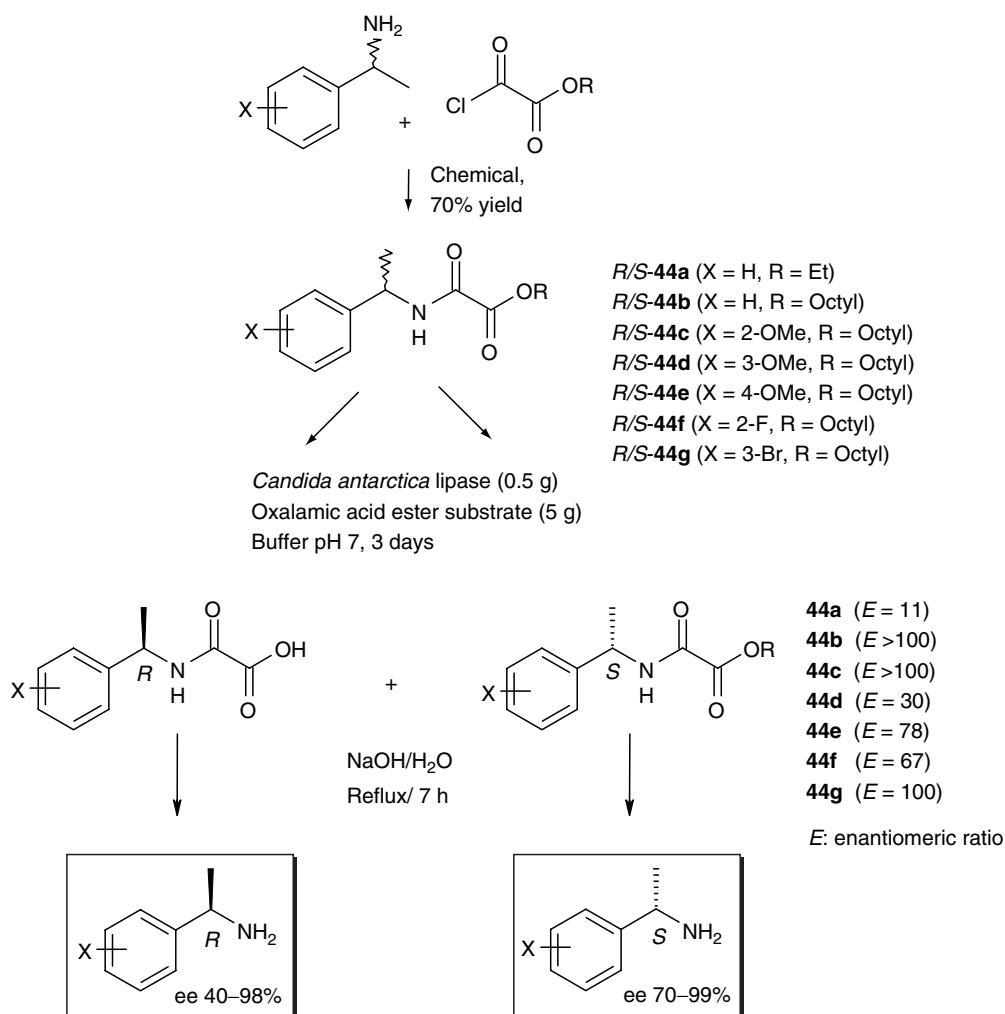
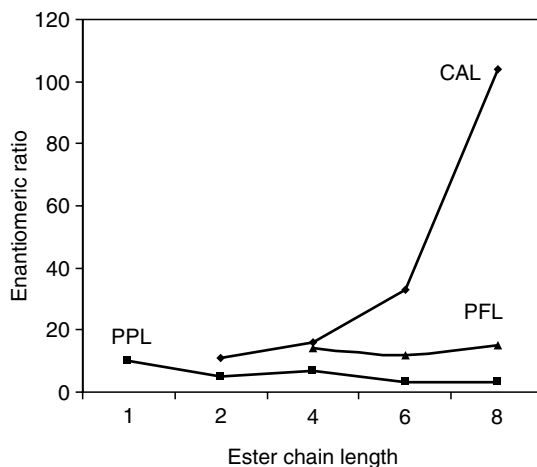
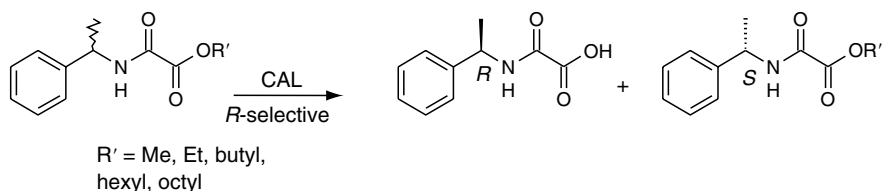


FIGURE 3.29 Resolution of amines by substrate engineering.

accessing both *R*- and *S*-isomers of primary and secondary amines in very high optical purities [102] (ee up to 99%).

3.6.1.2 Deracemization of Amino Acids and Amines

Based on the original reports by Hafner and Wellner [108] and Soda and coworkers [109,110] we envisaged a deracemization approach where a racemic amino acid is oxidized by an



CAL: *Candida antarctica* lipase (*R*-selective)
PFL: *Pseudomonas fluorescens* lipase (*S*-selective)
PPL: Porcine pancreatic lipase (*S*-selective)

FIGURE 3.30 Effect of ester chain length on enantioselectivity of lipase-catalyzed hydrolysis of oxalamic esters of 1-phenylethylamine.

enantioselective oxidase, which converts one enantiomer to the corresponding achiral imine. Addition of a nonselective chemical reducing agent to the mixture results in the reduction of imine back to 1:1 mixture of the amino acid. If this process is allowed to undergo a sufficient number of cycles, the resulting amino acid eventually reaches optical purity—yields can approach 100% if the imine can be efficiently reduced before it undergoes hydrolysis to the ketone (Figure 3.32). A significant limitation of these early studies, however, was the use of sodium borohydride (NaBH_4), which reacts with water and is easily decomposed at low and neutral pH, thus requiring large excesses to be added that can lead to deactivation of the oxidase enzyme [111].

In collaboration with Edinburgh University, United Kingdom, we developed a deracemization process [103] for acyclic/alicyclic α -amino acids and β -substituted α -amino acids using a combination of *R*-selective D-amino acid oxidases (D-AAO: porcine kidney, *Trigonopsis variabilis* [112]), and L-AAO (*Proteus myxofaciens* [113]; *Cellulomonas cellulans* [114]) (Figure 3.32 and Figure 3.33). In this approach amino acids (prepared in racemic forms and as a mixture of diastereomers from the corresponding racemic aldehydes by the Strecker reaction [115]) in buffer solutions, containing the appropriate chemical reducing agents, were incubated with D- or L-AAO and reactions were monitored by chiral HPLC. As an alternative to NaBH_4 , we used NaCNBH_3 , Pd/C- HCO_2NH_4 catalytic transfer hydrogenation [116], and amine boranes [117] ($\text{NH}_3:\text{NH}_3$) in combination with either commercially available D-AAO or microbial L-AAO grown in fermenters; for example, *P. myxofaciens* L-AAO, which has

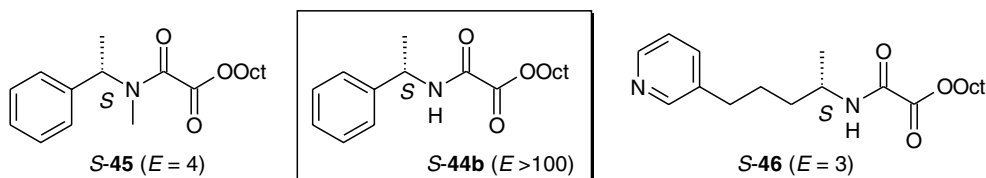


FIGURE 3.31 Effect of amino group substitution on enantioselectivity of *Candida antarctica* lipase (CAL)-catalyzed hydrolysis of octyl oxalamic ester derivatives.

been overexpressed in *E. coli* [113], was used either as a whole cell biocatalyst or as partially disrupted cells. A variety of acyclic (L-2-amino-4-Z-aminobutyric acid, L-lysine, D- α -aminobutyric acid, D-allylglycine, D-methionine, D-leucine), and alicyclic (D- and L-phenylalanine, L-piperazine-2-carboxylic acid, D-cyclopentyl glycine, L-proline) α -amino acids, as well as β -substituted α -amino acids (L-2-amino-3-methylhexanoic acid, L-2- β -methyl phenylalanine) were produced in very high chemical and optical yields (ee > 99%, yield 60 to 95%) (Figure 3.33).

Similarly, the same deracemization principle was applied to produce chiral amines, in high optical and chemical yields, using an enantioselective monoamine oxidase (MAO) coupled with nonselective chemical reducing agents [104,105] (Figure 3.34). Amine oxidases are classified mechanistically into two distinct groups. Type-I are copper-dependent and require topa quinone as a co-factor, whereas type-II are flavin-dependent. With type-I enzymes, the imine remains enzyme-bound and is not released as a free intermediate; these enzymes are therefore unlikely to be suitable for deracemizations. Type-II oxidases are more closely related to the D- and L-amino acid oxidases, in that they have a bound flavin unit and generate “free” imine [118]; known sources include bovine liver [119] and *Aspergillus niger* [120]. Schilling and Lerch [120,121] reported the cloning and expression of a type-II monoamine oxidase from *A. niger* (MAO-N), and subsequently Sablin *et al.* [122] purified the enzyme to homogeneity for substrate specificity and kinetic studies. The enzyme was very active toward simple aliphatic amines (amylamine, butylamine) but was also active, albeit at a much lower rate, toward benzylamine. In our experiments MAO-N showed very low but detectable activity toward L-(S)- α -methylbenzylamine with even slower oxidation of the D-(R)-enantiomer; the enzyme was only partially enantioselective. We therefore embarked on a program to improve both the catalytic activity and enantioselectivity using *in vitro* evolution methods [104]. A library of variants was generated by randomly mutating the plasmid containing MAO-N gene by using the *E. coli* XL1-Red mutator strain [105].

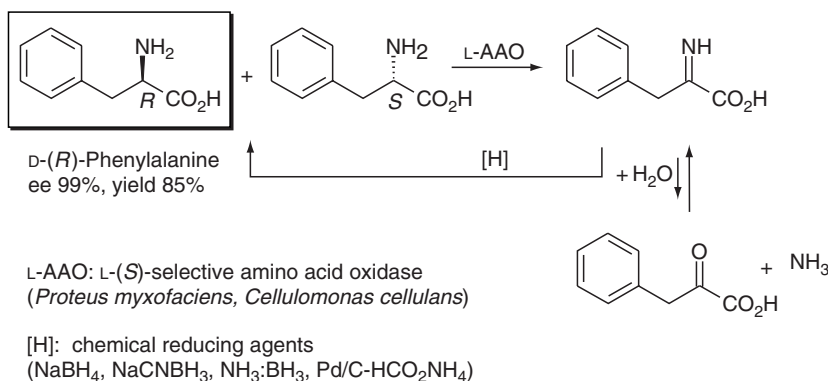
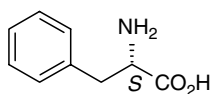
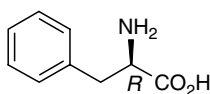


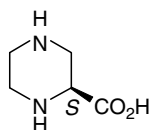
FIGURE 3.32 Deracemization of (±)- α -amino acids using S-selective L-amino acid oxidases coupled with chemical reducing agents.



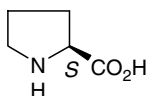
L-(*S*)-Phenylalanine
ee > 99%, yield 80%
Porcine kidney D-AAO
NaCNBH₃



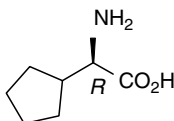
D-(*R*)-Phenylalanine
ee 99%, yield 85%
Proteus myxofaciens L-AAO
NH₃:BH₃



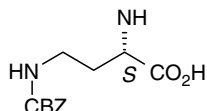
L-(*S*)-Piperazine-2-carboxylic acid
ee 99%, yield 95%
Porcine kidney, *Trigonopsis variabilis* D-AAO
Pd/C-HCO₂NH₄



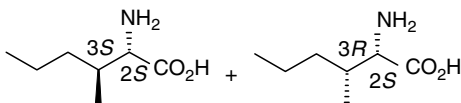
L-(*S*)-Proline
ee > 99%, yield 94%
Porcine Kidney D-AAO
NaCNBH₃



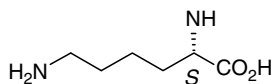
D-(*R*)-Cyclopentyl glycine
ee 94%, yield 80%
Proteus myxofaciens L-AAO
NH₃:BH₃



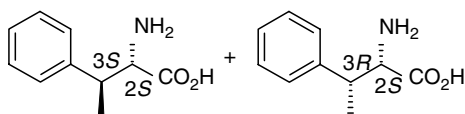
L-(*S*)-2-Amino-4-Z-aminobutyric acid
ee > 99%, yield 85%
Trigonopsis variabilis D-AAO
NaCNBH₃



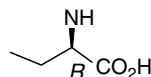
Isomers of L-(2*S*)-2-amino-3-methylhexanoic acid
ee 99%, yield 90%
Trigonopsis variabilis D-AAO, NaBH₄, NaCNBH₃



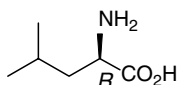
L-(*S*)-lysine
ee 98%, yield 95%
Trigonopsis variabilis D-AAO, NaCNBH₃



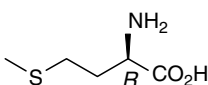
Isomers of L-(2*S*)-β-methyl phenylalanine
ee 99%, yield 85%
Trigonopsis variabilis D-AAO, NaCNBH₃



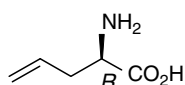
D-(*R*)-α-aminobutyric acid
ee 90%, yield 60%
Proteus myxofaciens L-AAO, NH₃:BH₃



D-(*R*)-leucine
ee > 99%, yield 80%
Proteus myxofaciens L-AAO
NH₃:BH₃



D-(*R*)-methionine
ee > 99%, yield 84%
Proteus myxofaciens L-AAO
NaCNBH₃, NH₃:BH₃



D-(*R*)-allylglycine
ee > 99%, yield 79%
Proteus myxofaciens L-AAO
NaCNBH₃, NH₃:BH₃

FIGURE 3.33 Glaxo Wellcome's deracemization process for production of optically pure natural and unnatural amino acids using D- or L-amino acid oxidases (AAO). (From Enright, A. and Mahmoudian, M., Glaxo Wellcome R&D, UK, unpublished work, 2001.)

An *S*-selective MAO-N mutant (Asn336Ser) was identified which showed significantly improved catalytic activity (47-fold) and enantioselectivity (sixfold) toward L-(*S*)- α -methylbenzylamine compared with the wild-type enzyme; enantioselectivity of the mutant (L- vs. D-isomer) was 100:1 compared with 17:1 for the wild-type enzyme [104]. This was confirmed by chiral HPLC where complete oxidation of L-(*S*)- α -methylbenzylamine was observed after 24 h, whereas no detectable conversion of the corresponding D-isomer was evident. Several reducing agents were screened (NaBH_4 , NaCNBH_3 , $\text{Pd/C-HCO}_2\text{NH}_4$, $\text{NH}_3:\text{NH}_3$) with 1 mM DL- α -methylbenzylamine using the mutant MAO-N; best conditions were observed with $\text{NH}_3:\text{NH}_3$, which afforded D-(*R*)- α -methylbenzylamine (ee 93%, yield 77%) (Figure 3.34). Using the mutant MAO-N we studied its substrate specificity toward a variety of alicyclic and acyclic primary and secondary amines using a high-throughput plate format; rate of oxidations were monitored by measuring H_2O_2 formation using a colorimetric enzyme assay [104] (Figure 3.34 and Figure 3.35). The wild-type MAO-N was inactive in most parts toward the amines studied but was most active toward simple straight chain amines, and showed very poor activity for the more sterically demanding branched amines. In sharp contrast, however, the mutant MAO-N (Asn336Ser) showed good activity toward branched acyclic amines, substituted α -methylbenzylamines ($\text{X} = \text{OMe}$, Me, Br, NO_2), alicyclic amines, and the secondary amine 1-methyltetrahydroisoquinoline [104] (Figure 3.35). In addition to showing a broad substrate specificity, the mutant MAO-N was highly *S*-selective giving the D-(*R*)-amine products in high optical and chemical yields [104] (Figure 3.34). Working with the wild-type MAO-N we found that, using site-directed mutagenesis, the identity of mutation at position 348 greatly influenced the efficiency of expression. We therefore used the mutant MAO-N (Asn336Ser) to introduce a second mutation (Met348Lys), by site-directed mutagenesis, which combined the catalytic enhancement of the Asn336Ser mutation with an increase in expression [104]. The activity of the double mutant enzyme (Asn336Ser, Met348Lys) was then examined with a variety of amine

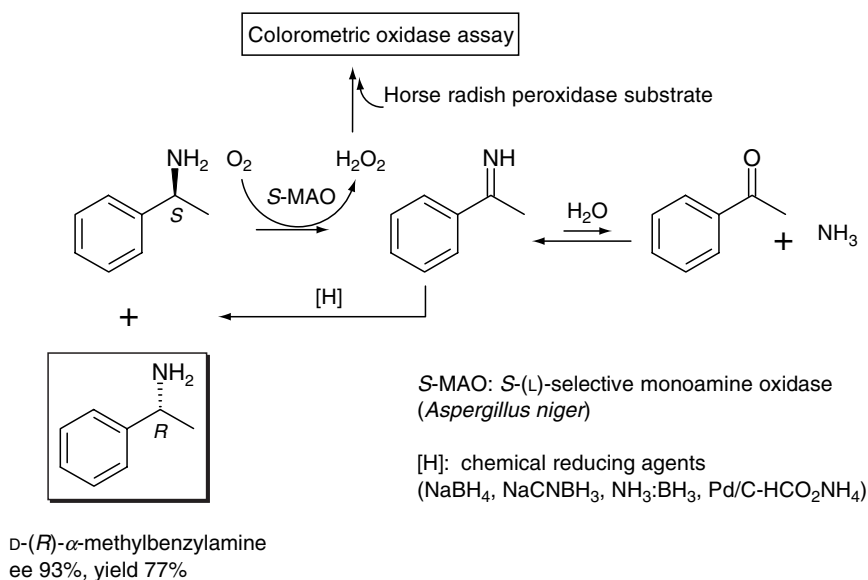


FIGURE 3.34 Deracemization of (\pm)-amines using an *S*-selective oxidase coupled with chemical reducing agents.

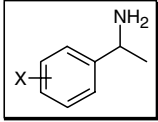

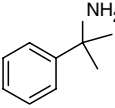
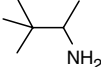
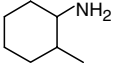
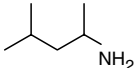
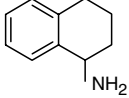
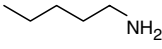
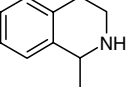
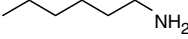
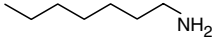
Alicyclic amines	Acyclic amines
 <p> 100%, X = H, <i>alpha</i>-methylbenzylamine 81%, X = OMe, 1-(4-methoxyphenyl) ethylamine 28%, X = Me, 4-methylphenyl ethylamine 24%, X = Br, 4-bromophenyl ethylamine 91%, X = NO₂, methyl-4-nitrobenzylamine </p>	 <p>540%, 3-Methyl-2-butylamine</p>
 <p>0%, Bis-α-methylbenzylamine</p>	 <p>140%, 3,3-Dimethyl-2-butylamine</p>
 <p>43%, 2-Methylcyclohexylamine (<i>cis/trans</i>)</p>	 <p>180%, 1, 3-Dimethylbutylamine</p>
 <p>13%, 1, 2, 3, 4-Tetrahydro-1-naphthylamine</p>	 <p>2520%, <i>n</i>-amylamine</p>
 <p>67%, 1-Methyltetrahydroisoquinoline</p>	 <p>1860%, <i>n</i>-hexylamine</p>
	 <p>1210%, <i>n</i>-heptylamine</p>

FIGURE 3.35 Glaxo Wellcome's deracemization process for production of optically pure amines using the *S*-selective mutant (Asn336Ser) monoamine oxidase from *Aspergillus niger*. (From Alexeeva, M., Enright, A., Mahmoudian, M., et al., PCT Int. Appl. WO 03/080855, 2003.) All rates are measured relative to L-(*S*)- α -methylbenzylamine (100%). Rate of oxidation was monitored by measuring H₂O₂ production using a colorimetric oxidase assay.

substrates (rates compared to L-(*S*)- α -methylbenzylamine set at 100%, [Figure 3.36](#)); the enzyme demonstrated exquisite *S*-selectivity in almost every case (ee 90 to 99%) [104].

In summary, we have developed two deracemization processes for production of (i) a variety of optically pure natural and unnatural L- and D- α -amino acids as well as β -substituted α -amino acids using D- or L-amino acid oxidases [103]; and (ii) a range of alicyclic and branched acyclic optically pure primary and secondary D-(*R*)-amines using the *S*-selective mutant amine oxidase from *A. niger* obtained by directed evolution [104,105]. In these approaches, the oxidase enzyme is coupled with nonselective chemical reducing agents (NaBH₄, NaCNBH₃, NH₃:BH₃, Pd/C-HCO₂NH₄) to afford amino acids and amines in high chemical and optical purities (ee > 99%, yield 70 to 99%) [103–105].

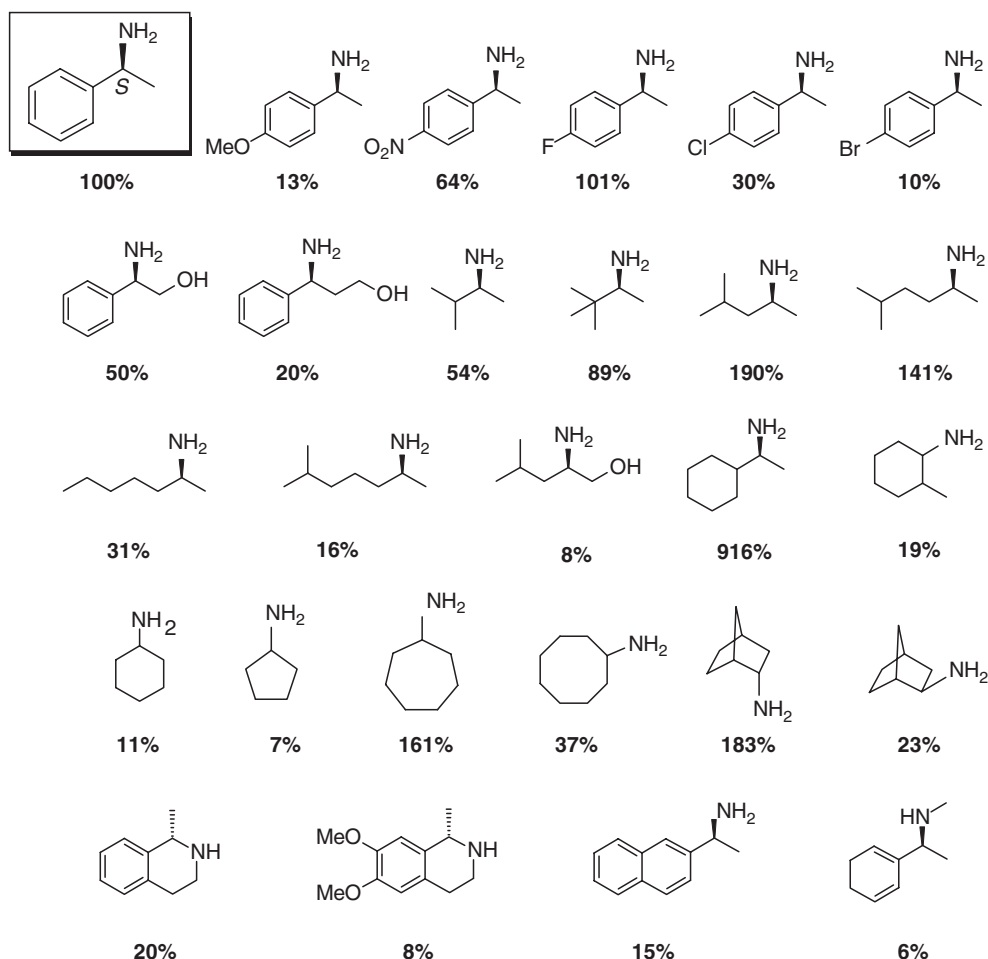


FIGURE 3.36 Glaxo Wellcome's process for production of optically pure amines using the *S*-selective double mutant (Asn336Ser, Met348Lys) monoamine oxidase. (From Alexeeva, M., Enright, A., Mahmoudian, M., et al., PCT Int. Appl. WO 03/080855, 2003.) All rates are measured relative to L-(*S*)- α -methylbenzylamine (100%). Rate of oxidation was monitored by measuring H_2O_2 production using a colorimetric oxidase assay.

3.6.2 HIGH-THROUGHPUT SCREENING USING INFRARED THERMOGRAPHY

At Glaxo Wellcome we often screened isolated enzymes, recombinant microorganisms, and chiral ligands for enantioselective transformations. For HTS, the bottleneck is usually in the speed of chiral analyses involving laborious development of chiral assays. We were therefore particularly interested in developing new approaches for rapid determination of ee. This is, however, frequently hampered by the availability of a suitable chiral assay; chiral HPLC/GC and chiral shift NMR are the most preferred techniques but these methods can suffer from being too time-consuming to develop. Other approaches used by several groups include fluorescence [123–125], mass spectroscopy [126], capillary electrophoresis [127], and circular dichroism [128]. Among these methods infrared thermography (IRT) has

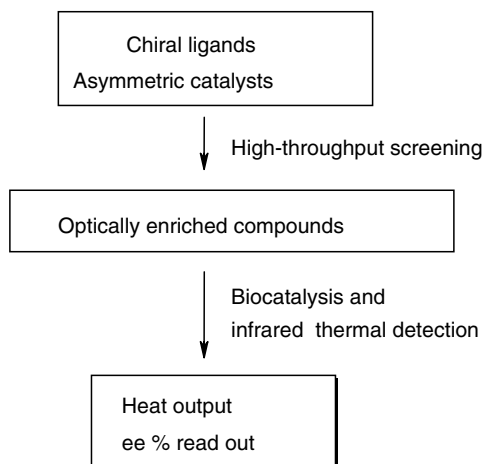


FIGURE 3.37 Infrared thermography (IRT) for high-throughput screening (HTS) of catalysts for asymmetric transformations.

attracted considerable interest in recent years because noninvasive thermal imaging of chemical reactions can be performed through detection of emitted infrared radiations [129]. Acceleration of drug screening is one of the potential applications of IRT in the pharmaceutical industry. Reetz et al. [130–131] highlighted the use of thermal imaging for screening of enantioselective biocatalysts and observed a differential heat output during the thermal imaging of lipase-catalyzed acylation of chirally pure *R*- and *S*-alcohols [130]. We therefore envisaged a high-throughput chiral screening approach based on thermal detection of enzyme-catalyzed kinetic resolutions of racemic mixtures and went on to develop this further [132] (Figure 3.37).

In this approach immobilized CAL was used in 96-well microtitre plates containing solutions of racemic 1-phenylethanol *Rac*-**47** (ee 1%, prepared by sodium borohydride reduction of acetophenone) and *R*-**47** (ee 86%, prepared by Corey–Bakshi–Shibata (CBS) reduction of acetophenone) (Figure 3.38a, second row). Standard solutions of the various mixtures of optically pure *R*- and *S*-**47** (0 to 100% *R*- or *S*-isomer) were used for determining ee values (Figure 3.38a, first row). Temperature changes were monitored periodically with an infrared camera (thermal imaging) and reactions were started by adding solutions of vinyl acetate in toluene to each well. Before start of reactions, cold spots (blue) were evident due to the endothermic process of solvent evaporation (Figure 3.38a). Subsequently, we observed differential heat outputs (yellow) corresponding to various concentrations of the *R*-enantiomer (Figure 3.38b, first row). Similarly, wells containing either *Rac*-**47** or *R*-**47** (prepared by chemical reductions) showed elevated heat outputs (Figure 3.38b, second row). CAL acylation of *Rac*-**47** was therefore shown to be *R*-enantioselective (the *R*-isomer is preferentially acylated leaving behind the corresponding *S*-isomer, Figure 3.38). This suggested that an excellent *R*-enantioselectivity was also obtained after CBS reduction [133] of acetophenone (ee 86% by chiral HPLC) (Figure 3.38). The temperature changes from enzymic reactions were quantified by measuring heat output as a function of time, and the areas under each temperature curve could now be used to calculate ee values of *Rac*-**47** (ee 8%) and optically enriched *R*-**47** (ee 94%). These corresponded very well with ee values measured by chiral HPLC (1 vs. 86% respectively) [132]. Similarly we applied IRT determination of ee to other substrates such as 2-hexanol **48** and 1-phenylethylamine **49** (Figure 3.39). The time-resolved

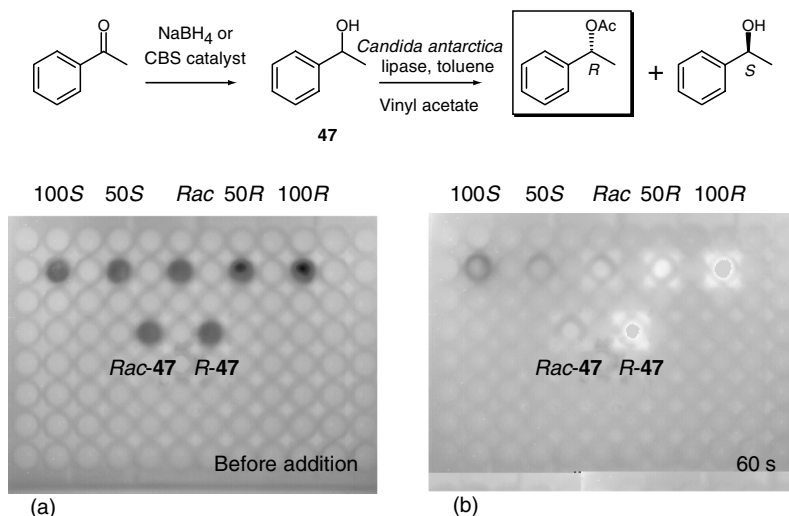


FIGURE 3.38 (See color insert following page 526) Time-resolved thermal imaging of *Candida antarctica*-catalyzed acylation of 1-phenylethanol **47**. NaBH₄ reduction of acetophenone afforded *Rac*-**47** (ee 1%, chiral HPLC), whereas Corey–Bakshi–Shibata (CBS) reduction gave *R*-**47** (ee 86%, chiral HPLC). Colors correspond to the following temperatures: (a) at time zero—black (12.5 to 13.5°C), purple (15 to 16°C), pink (17 to 18°C), orange (18.5 to 19.5°C); (b) after 60 s—orange (18.5 to 19°C), yellow (19.5 to 20°C), white (20.5°C), gray (21.4 to 21.6°C).

IRT temperature profile (standard curves) for *C. antarctica* at various mixtures of optically pure **48** or **49** (100*S*–100*R*) was obtained in microtitre plates, which gave good linear correlations with the observed ee values. The data were analyzed using the statistical software package Minitab [132].

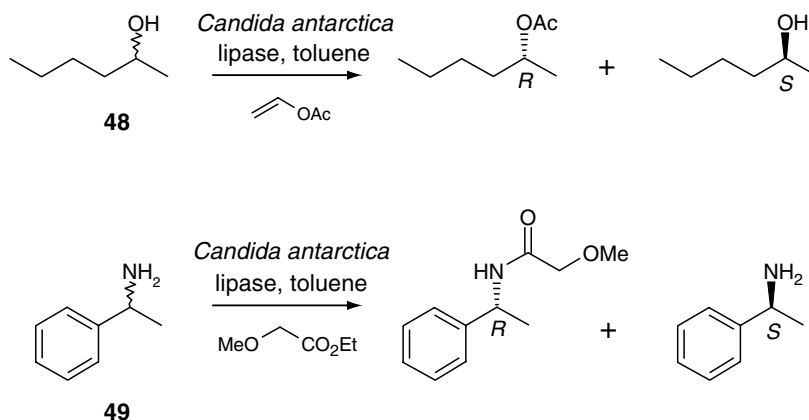


FIGURE 3.39 *Candida antarctica*-catalyzed acylation of 2-hexanol **48** and 1-phenylethylamine **49** using infrared thermography (IRT) for determination of enantiomeric excess (ee). Either vinyl acetate or ethyl methoxyacetate was used as acylating agents. Standard curves for ee determination by IRT were obtained. Temperature areas (s.°K), second x Kelvin, representing various mixtures of either **48** or **48** isomers (100*S*–100*R*) were obtained in microtitre plates, which gave good linear correlations with the observed ee values. The data were analyzed using the statistical software package Minitab. (From Millot, N., Borman, P., Mahmoudian, M., et al., *Org. Process R&D.*, 6, 463–470, 2002.)

Having these encouraging results in hand, we decided to extend the application of IRT to rapid screening of a variety of racemic secondary alcohols in a 96-well plate using CAL (Table 3.1). However, an important criterion of the HTS campaign is speed, i.e., the ability to rapidly identify the substrates of interest for further study. Although desirable, it is unrealistic to expect an HTS process to deliver completely reliable and reproducible data for every substrate. With this in mind we proceeded with analysis of thermal imaging pictures of the microtitre plate, taken at regular time intervals, which showed that strongest heat output was immediately observed for alcohol G9 having a tertiary amino group in γ -position [132] (Table 3.1). Significant temperature rises were also seen after 10 s for substrates with a tertiary amino group at α -position (E9 and F9). For comparison, heat outputs from reactions of similar alcohols not containing any basic functionality (e.g., A4, B4, D4, B12, and G11) were detected much later. This may suggest that kinetics of acylation of alcohols E9–G9 could be substantially different than for reactions with alcohols having no basic functionality. Attempts to obtain a more accurate readout for each well proved to be more tricky; interestingly, despite showing small temperature rises, no reaction at all was detected in 11 wells (B1, H3, F6, A7, B7, C9, D9, B11, C11, E11, D12) as judged by GC, highlighting a potential limitation of IRT. For example, the highest temperature rise was 3.1°K (Kelvin) for a well with no reaction. This was therefore used as background reading; 46 wells gave a temperature rise under 3.1°K, whereas the remaining 50 wells showed a temperature rise above 3.1°K. This detection rate is typical for HTS, where the emphasis is on rapid detection of interesting wells for further study rather than an exhaustive assessment of each substrate. Furthermore, although chiral analyses (chiral GC) of selected wells showed that in some cases acylations were indeed enantioselective, this did not correspond well with the generated heat outputs (Table 3.2). These findings on the substrate specificity of CAL for acylations of alcohols are also consistent with previously reported data on its active site [134].

































































































In summary, IRT is a novel technique that can be used to screen a potentially large number of asymmetric catalysts or substrates in a high-throughput fashion, and also for rapid determination of ee. Here we used IRT as a fast, simple, and practical method for initial screening of the substrate specificity of CAL for the generation of chiral secondary alcohols and amines using a 96-well microtitre plate format. The potential drawback of IRT was also highlighted when detecting the heat output in a 96-well format, and the apparent lack of correlation between the observed heat output and enantioselectivity in some cases. However, we believe that IRT can provide a crude but practical initial method, when screening large numbers of catalysts and/or substrates, to rapidly identify a particular structural class that can be further accurately analyzed by conventional methods. For example, the data generated by IRT for the whole plate (96 substrates) took only 15 min—several orders of magnitude quicker than analysis by GC or HPLC.

3.7 SUMMARY

The use of biotech tools to discover new molecules, and to develop and improve environmentally friendly processes for their production, is widespread in pharmaceutical, agrochemical, chemical, and biotech sectors as well academia. During 1989–2002, we at Glaxo Wellcome had an active biotech program, routinely utilizing enzymes and recombinant microorganisms in most aspects of our drug discovery and development pipeline, to (i) generate complex templates, which would otherwise be difficult to synthesize chemically, for use in combinatorial libraries; (ii) produce natural products and secondary metabolites through fermentation processes; (iii) functionalize novel molecules for structure activity relationship (SAR) to improve activity, potency, and/or water solubility; (iv) use microbial

TABLE 3.1

Rapid Screening of Racemic Secondary Alcohols in 96-Well Plate Format Using *Candida antarctica* Lipase (CAL) and Infrared Thermography (IRT)

H	G	F	E	D	C	B	A	
 3.5; 54	 5.1; 100	 3.4; 28	 2.1; 52	 1.9; 2	 2.4; 12	 2.8; 0	 6.4; 57	1
 2.5; 60	 4.8; 62	 3.9; 44	 2.9; 40	 3.9; 41	 3.1; 72	 3.8; 44	 7.1; 43	2
 1.9; 0	 4.2; 99	 4.3; 51	 4.5; 60	 5.2; 60	 4.2; 30	 4.5; 63	 85; 7	3
 4.9; 54	 4.6; 60	 4.9; 56	 5.2; 62	 5.4; 55	 6.2; 60	 6.6; 58	 5.7; 59	4
 1.8; 32	 1.7; 29	 2.8; 30	 2.8; 7	 4.3; 59	 4.4; 47	 4.0; 57	 4.0; 50	5
 3.1; 31	 2.7; 55	 3.1; 0	 2.8; 33	 3.2; 54	 2.8; 7	 3.5; 58	 4.0; 100	6
 1.6; 6	 3.0; 60	 4.4; 61	 4.0; 83	 3.1; 20	 2.5; 80	 2.5; 0	 2.6; 0	7
 2.1; 53	 2.1; 30	 3.0; 43	 3.5; 50	 3.4; 53	 2.9; 50	 2.5; 93	 3.2; 67	8
 2.3; 60	 6.8; 74	 3.4; 56	 3.1; 59	 2.8; 0	 2.9; 0	 3.1; 43	 3.6; 59	9
 3.1; 40	 3.2; 40	 4.5; 63	 2.0; 62	 2.6; 65	 4.1; 68	 5.1; 55	 5.0; 100	10
 2.7; 36	 3.4; 46	 6.0; 53	 2.4; 0	 2.2; 20	 1.8; 0	 2.7; 0	 3.4; 7	11
 3.6; 57	 2.4; 51	 4.2; 78	 2.5; 46	 1.7; 0	 2.6; 46	 2.6; 46	 2.8; 40	12

Note: Numbers are related to the temperature rise (°K) measured by IRT and conversions (%) measured by GC after 90 min. A–H and 1–12 represent rows of microtitre plate.

TABLE 3.2
Correlation between Enantiomeric Ratio (*E*) and the Observed
Temperature Rise (Kelvin, °K) by Thermal Imaging for Lipase-Catalyzed
Acylation of Selected Wells in Table 3.1

Wells	Conversion (%)	ee (%)	<i>E</i>	IRT (°K)
A8	67	0	1.0	3.2
C2	72	33	4.8	3.1
D2	41	24	1.9	3.9
D7	20	72	7.3	3.1
G8	30	7	1.2	2.1
G10	40	30	2.2	3.2
H10	40	52	4.4	3.1

Note: Conversions and enantiomeric excess (ee) determinations were carried out using chiral gas chromatography (GC).

IRT, infrared thermography.

models to generate metabolites that were used as standards in mammalian metabolism (impurity profiling); and (v) produce gram to tonne quantities of key enantiomerically pure intermediates for the synthesis of development compounds. Biocatalytic approaches were often considered at the very inception of a chemical route, for example, to bypass an environmentally “unfriendly” step (e.g., use of transitional metal oxidants such as potassium permanganate) or even to replace several chemical stages with a single enzymic step. Case studies reviewed in this chapter highlight the importance of close integration of biotech approaches at various stages of drug discovery and chemical development processes in pharma. The potential benefits of biotech tools will be fully realized as more companies adopt a similar strategy.

Biotechnology will grow in many sectors over the next 10 y and can no longer be ignored in certain product areas and market segments. A diverse set of tools will be needed with most new technologies being assembled at interface of traditional disciplines. In sharp contrast to the pharmaceutical and biotech sectors, surprisingly there are only a relatively few chemical products made through biotech approaches in the chemical sector, with some notable exceptions being acrylamide production and some commodity chemicals such as high-fructose corn syrup; overall this is proving to be a challenging space indeed. There are, however, a number of companies actively trying to penetrate the bio-based products market: (i) DuPont/Genencor/Staley (1,3-propane-diol biopolymer); (ii) Cargill Dow (polylactic acid biodegradable plastic); and (iii) Eastman/Genencor (vitamin-C process). At Rohm and Haas we are developing key capabilities and multiple business platforms at the intersection of biosciences and polymer sciences to explore, expand, and develop new market opportunities.

ACKNOWLEDGMENTS

This chapter is dedicated to Helen, Laura, and Yasmin who have supported me unfailingly. I gratefully acknowledge contributions of many colleagues from Glaxo’s Natural Products Discovery Department (1989–2002), and Glaxo Wellcome Operations for the scale-up of these bioprocesses.

REFERENCES

1. McKinsey & Company, Biotechnology in chemical industry, *The McKinsey Quarterly*, 2002.
2. Van Arnum, F., White biotechnology: the chemical industry's next challenge, *Focus*, 2003; fine chemicals/biopharmaceuticals, *Chemical Market Reporter*, 263: i24 pFR6–1, 2003.
3. Tamburini, B., Perboni, A., Rossi, T., et al., Preparation of 10-(1-hydroxyethyl)-11-oxo-1-azatricyclo[7.2.0.0.3,8]undec-2-ene-2-carboxylic acid derivatives as antibacterials, *Eur. Patent Appl.*, 0416953, 1991.
4. Stead, P., Marley, H., Mahmoudian, M., et al., Efficient procedures for the large-scale preparation of (+)-(1*S*, 2*S*)-*trans*-2-methoxycyclohexanol, a key chiral intermediate in the synthesis of tricyclic β -lactam antibiotics, *Tetrahedron Asymmetry*, 7(8), 2247–2250, 1996.
5. Laumen, K., Breitgoff, D., Seemayer, R., et al., Enantiomerically pure cyclohexanols and cyclohexane-1,2-diol derivatives; chiral auxiliaries and substitutes for (–)-8-phenylmenthol: a facile enzymatic route, *J. Chem. Soc. Chem. Commun.*, 148–150, 1989.
6. Basavaiah, D. and Krishna, P.R., Pig liver powder as biocatalyst: enantioselective synthesis of *trans*-2-alkoxycyclohexan-1-ols, *Tetrahedron*, 50, 10521–10530, 1994.
7. Honig, H. and Seuffer-Wasserthal, P., A general method for the separation of enantiomeric *trans*-2-substituted cyclohexanols, *Synthesis*, 1137–1140, 1990.
8. Halpern, B. and Westley, J.W., Chemical resolution of secondary alcohols, *Aust. J. Chem.*, 19, 1533–1538, 1966.
9. Averett, D.R., Koszalka, G.W., Fyfe, J.A., et al., 6-Methoxypurine arabinoside as a selective and potent inhibitor of varicella-zoster virus, *Antimicrob. Agents Chemother.*, 35, 851–857, 1991.
10. Lambe, C.U., Averett, D.R., Paff, M.T., et al., 2-Amino-6-methoxypurine arabinoside: an agent for T-cell malignancies, *Cancer Res.*, 55, 3352–3356, 1995.
11. Krenitsky, T.A., Averett, D.R., Moorman, A.R., et al., Method of treating T-Cell lymphoblastic leukaemia with *ara*-G nucleoside derivatives, *U.S. Patent Appl.*, 5492897, 1996.
12. Moorman, A.R., Chamberlain, S.D., Jones, L.A., et al., 5'-ester prodrugs of the varicella-zoster antiviral agent, 6-methoxypurine arabinoside, *Antiviral Chem. Chemother.*, 3, 141–146, 1992.
13. Eaddy, J., Glaxo Wellcome R&D, USA, unpublished work, 1997.
14. Mahmoudian, M., Eaddy, J., and Dawson M.J., Enzymatic acylation of 506U78: a powerful new anti-leukaemic agent, *Biotechnol. Appl. Biochem.*, 29, 229–233, 1999.
15. Gregson, M., Ayres, B.E., Ewan, G.B., et al., 2,6-Diaminopurine derivatives, *PCT Int. Appl.*, WO 9417090, 1994.
16. Isono, Y. and Hoshino, M., Nucleoside oxidase and assay method utilizing same, *U.S. Patent Appl.*, 5156955, 1992.
17. Isono, Y., Sudo, T., and Hoshino, M., Purification and reaction of a new enzyme, nucleoside oxidase, *Agric. Biol. Chem.*, 53, 1663–1669, 1989.
18. Isono, Y., Sudo, T., and Hoshino, M., Properties of a new enzyme, nucleoside oxidase, from *Pseudomonas maltophilia* LB-84, *Agric. Biol. Chem.*, 53, 1671–1677, 1989.
19. Misaki, H., Ikuta, S., and Matsunai, K., Nucleoside oxidase and process for making same, and process and kit for using same, *U.S. Patent Appl.*, 4385112, 1983.
20. Mahmoudian, M., Rudd, B.A.M., Cox, B., et al., A versatile procedure for the generation of nucleoside 5'-carboxylic acids using nucleoside oxidase, *Tetrahedron*, 54, 8171–8182, 1998.
21. Gallo, R.C., Sarin, P.S., Gelman, E.P., et al., Isolation of a human T-cell leukaemia virus in AIDS, *Science*, 220, 865–867, 1983.
22. Fauchi, A.S., Masur, H., Gelman, E.P., et al., NIH conference: the AIDS update, *Ann. Intern. Med.*, 102, 800–813, 1985.
23. Coates, J.A.V., Cammack, N., Jenkinson, H.J., et al., (–)-2'-Deoxy-3'-thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus Type 1 and Type 2 replication *in vitro*, *Antimicrob. Agents Chemother.*, 36, 733–739, 1992.
24. Coates, J.A.V., Cammack, N., Jenkinson, H.J., et al., The separated enantiomers of 2'-deoxy-3'-thiacytidine (BCH189) both inhibit human immunodeficiency virus replication *in vitro*, *Antimicrob. Agents Chemother.*, 36, 202–205, 1992.
25. Cameron, J.M., Collis, P., Daniel, M., et al., Lamivudine, *Drugs of the Future*, 18, 319–323, 1993.

26. Francis, C.E., Hunter, A., Berney, J.J., et al., *In vitro* myelotoxicity studies with 3TC[®]: 8th International conference on AIDS and 3rd STD world congress (Amsterdam), Abs. no. PuA. 6027, 1992.
27. Storer, R., Clemens, I.R., Lamont, B., et al., The resolution and absolute stereochemistry of the enantiomers of *cis*-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (BCH189): equipotent anti-HIV agents, *Nucleosides Nucleotides*, 12, 225–236, 1993.
28. Mahmoudian, M., Baines, B.S., Drake, C.S., et al., Enzymatic production of optically pure 3TC[®] (Lamivudine): a potent anti-HIV agent. *Enzyme Microb. Technol.*, 15, 749–755, 1993.
29. Hammer-Jespersen, K., Nucleoside metabolism, in *Metabolism of nucleotides, nucleosides, and nucleobases in microorganisms*, Munch-Petersen, A., Ed., Academic Press, London, 1983, pp. 203–258.
30. Cousins, R.P.C., Mahmoudian, M., and Youds, P.M., Enzymatic resolution of oxathiolane intermediates: an alternative approach to the anti-viral agent lamivudine (3TC[®]), *Tetrahedron Asymmetry*, 6, 393–396, 1995.
31. Mahmoudian, M. and Dawson, M.J., Chemoenzymatic production of the antiviral agent Epivir, in *Biotechnology of antibiotics*, 2nd Ed., Strohl, W.R., Ed., Marcel Dekker Inc., New York, 1997, pp. 753–777.
32. Daluge, S.M., Therapeutic nucleosides, Eur. Patent Appl., 0434450, 1991.
33. Daluge, S.M., Good, S.S., Faletto, M.B., et al., 1592U89 a novel carbocyclic nucleoside analogue with potent selective anti-HIV activity, *Antimicrob. Agents Chemother.*, 41, 1082–1093, 1997.
34. Evans, C.T. and Roberts, S.M., Chiral azabicycloheptanone and a process for their preparation, Eur. Patent Appl., 0424 064 B1, 1991.
35. Evans, C.T., Roberts, S.M., Shoberu, K.A., et al., Potential use of carbocyclic nucleosides for the treatment of AIDS: chemoenzymatic synthesis of the enantiomers of carbovir, *J. Chem. Soc. Perkin Trans.*, 1, 589–592, 1992.
36. Mahmoudian, M., Lowdon, A., Jones, M.F., et al., A practical enzymatic procedure for the resolution of *N*-substituted 2-azabicyclo[2.2.1]hept-en-3-one. *Tetrahedron Asymmetry*, 10, 1201–1206, 1999.
37. Novozyme: Savinase 6T, Lipolase 100T, Novo Nordisk enzyme data sheet, 1996.
38. Dawson, M.J., Mahmoudian, M., and Wallis, C., Process for the enantioselective hydrolysis of *N*-derivatised lactam, Int. Appl. No. PCT/EP98/05291, 1999.
39. Von Itzstein, M., Wu, W.Y., Kok, G.B., et al., Rational design of potent sialidase-based inhibitors of influenza virus replication, *Nature*, 363, 418–423, 1993.
40. Schauer, R., Chemistry metabolism and biological function of sialic acids, *Adv. Carbohydr. Chem. Biochem.*, 40, 131–234, 1982.
41. De Ninno, M.P., The synthesis and glycosidation of *N*-acetyl-D-neuraminic acid, *Synthesis*, 8, 583–593, 1991.
42. Juneja, L.R., Koketsu, M., Nishimoto, K., et al., Large-scale preparation of sialic acid from chalaza and egg-yolk membrane, *Carbohydr. Res.*, 214, 179–186, 1991.
43. McGuire, E.J. and Binkley, S.B., The structure and chemistry of colominic acid, *Biochemistry*, 3, 247–251, 1964.
44. Uchida, Y., Tsukada, Y., and Sugimori, T., Improved microbial production of colominic acid, a homopolymer of *N*-acetyl-D-neuraminic acid, *Agric. Biol. Chem.*, 37, 2105–2110, 1973.
45. Marugane-Shoyu, K.K., Preparation of colominic acid, Jap. Patent Appl. 144989, 1989.
46. Spivak, C.T. and Roseman, S., Preparation of *N*-acetyl-D-mannosamine (2-acetamido-2-deoxy-D-mannose) and D-mannosamine hydrochloride (2-amino-2-deoxy-D-mannose), *J. Am. Chem. Soc.*, 81, 2403–2404, 1959.
47. Kragl, U., Gygas, D., Ghisalba, O., et al., Enzymatic two-step synthesis of *N*-acetyl-D-neuraminic acid in the enzyme membrane reactor, *Angew Chem. Int. Ed. Engl.*, 30, 827–828, 1991.
48. Auge, C., David, S., and Gautheron, C., Synthesis with immobilized enzyme of the most important sialic acid, *Tetrahedron Lett.*, 25, 4663–4664, 1984.
49. Bednarski, M.D., Chenault, H.K., Simon, E.S., et al., Membrane-enclosed enzymatic catalysis (MEEC): a useful, practical new method for the manipulation of enzymes in organic synthesis, *J. Am. Chem. Soc.*, 109, 1283–1285, 1987.

50. Kim, M.J., Hennen, W.J., Sweers, H.M., et al., Enzymes in carbohydrate synthesis: *N*-acetyl-D-neuraminic acid aldolase catalyzed reactions and preparations of *N*-acetyl-2-deoxy-D-neuraminic acid derivatives, *J. Am. Chem. Soc.*, 110, 6481–6486, 1988.
51. Tsukada, Y. and Ohta, Y., Process for producing *N*-acetyl-D-neuraminic acid, Eur. Patent Appl. 0578825A1, 1992.
52. Dawson, M.J., Noble, D., and Mahmoudian, M., Process for the preparation of *N*-acetyl-D-neuraminic acid, PCT W0 9429476, 1994.
53. Mahmoudian, M., Noble, D., Drake, C.S., et al., An efficient process for production of *N*-acetylneuraminic acid using *N*-acetylneuraminic acid aldolase, *Enzyme Microb. Technol.*, 20, 393–400, 1997.
54. Starkey, I.D., Mahmoudian, M., Noble, D., et al., Synthesis and influenza virus sialidase inhibitory activity of the 5-desacetoamido analogue of 2,3-didehydro-2,4-dideoxy-4-guanidiny-*N*-acetylneuraminic acid (GG167), *Tetrahedron Lett.*, 36, 299–302, 1995.
55. Woods, J.M., Bethell, R.C., Coates, J.A.V., et al., 4-Guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid is a highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses *in vitro*, *Antimicrob. Agents Chemoth.*, 37, 1473–1479, 1993.
56. Cermak, R.C. and Vince, R., (\pm)-4 β -Amino-2 α ,3 α -dihydroxy-1 β -cyclopentanemethanol hydrochloride: carbocyclic ribofuranosylamine for the synthesis of carbocyclic nucleosides, *Tetrahedron Lett.*, 22, 2331–2332, 1981.
57. Marquez, V.E. and Lim, M., Carbocyclic nucleosides, *Med. Res. Rev.*, 6, 1–40, 1986.
58. Borthwick, A.D., Butt, S., Biggadike, K., et al., Synthesis and enzymatic resolution of carbocyclic 2'-*ara*-fluoro-guanosine: a potent new anti-herpetic agent, *J. Chem. Soc. Chem. Commun.*, pp. 656–658, 1988.
59. Vince, R. and Brownell, J., Resolution of racemic carbovir and selective inhibition of human immunodeficiency virus by the (–)-enantiomer, *Biochem. Biophys. Res. Commun.*, 168, 912–916, 1990.
60. Balzarini, J., Baumgartner, H., Bodenteich, M., et al., Synthesis and anti-viral activity of the enantiomeric forms of carba-5-iodo-2'-deoxyuridine and carba-(*E*)-5-(2-bromovinyl)-2'-deoxyuridine, *J. Med. Chem.*, 32, 1861–1865, 1989.
61. Balzarini, J., Metabolism and mechanism of anti-retroviral action of purine and pyrimidine derivatives, *Pharm. World Sci.*, 16, 113–126, 1994.
62. Mahmoudian, M., Baines, B.S., Dawson, M.J., et al., Resolution of 4-amino-cyclopentanecarboxylic acid methyl esters using hydrolytic enzymes, *Enzyme Microb. Technol.*, 14, 911–916, 1992.
63. Krenitsky, T.A., Koszalka, G.W., Tuttle, J.V., et al., An enzymatic synthesis of purine D-arabinonucleosides, *Carbohydrate Res.*, 97, 139–146, 1981.
64. Herdewijn, P., Balzarini, J., de Clercq, E.A., et al., Resolution of aristeromycin enantiomers, *J. Med. Chem.*, 28, 1385–1386, 1985.
65. Secrist, J.A., Montgomery, J.A., Shealy Y.F., et al., Resolution of racemic carbocyclic analogues of purine nucleosides through the action of adenosine deaminase: antiviral activity of the carbocyclic 2'-deoxyguanosine enantiomers, *J. Med. Chem.*, 30, 746–749, 1987.
66. Hoong, L.K., Strange, L.E., Liotta, D.C., et al., Enzyme-mediated enantioselective preparation of pure enantiomers of the anti-viral agent 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC) and related compounds, *J. Org. Chem.*, 57, 5563–5565, 1992.
67. Cameron, J.M., New anti-herpes drugs in development, *Rev. Med. Virol.*, 3, 225–236, 1993.
68. Boehme, R.E., Berford, A., Hart, G.J., et al., GR95168X (chiral carbocyclic BVdU) is highly effective against simian *Varicella* virus infections of African green monkeys, *Antiviral Res.*, 23, 97–98, 1994.
69. Sicsic, S., Ikbali, M., and Le Goffic, F., Chemoenzymatic approach to carbocyclic analogues of ribonucleotides and nicotinamide ribose, *Tetrahedron Lett.*, 28, 1887–1888, 1987.
70. Csuk, R. and Doerr, P., Bioanalytical transformations: the 4-acetamido-cyclopent-2-ene carboxylate route revisited; synthesis of (+)- and (–)-aristeromycin, *Tetrahedron*, 51, 5789–5798, 1995.
71. Borthwick, A.D., Crame, A.J., Mahmoudian, M., et al., A short, convergent synthesis of two chiral anti-viral agents (+)-carbocyclic 2'-deoxy-5-[(*E*)-2-bromovinyl]uridine and (+)-carbocyclic 2'-deoxyguanosine, *Tetrahedron Lett.*, 36, 6929–6932, 1995.

72. Archelas, A. and Morin, C., Biohydroxylation of a 2-azabicyclo[2.2.1]heptane, *Tetrahedron Lett.*, 25, 1277–1278, 1984.
73. Dawson, M.J., Mahmoudian, M., Rudd, B.A.M., et al., Biosynthesis and biotransformation in the preparation of nucleoside analogues, in *Chiral Europe*, 1996, pp. 35–38.
74. White, E.L., Parker, W.B., Macy, L.J., et al., Comparison of the effect of carbovir, AZT, and dideoxynucleoside triphosphates on the activity of human immunodeficiency virus reverse transcriptase and selected human polymerases, *Biochem. Biophys. Res. Commun.*, 161, 393–398, 1989.
75. Carter, S.G., Kessler, J.A., and Rankin, C.D., Activities of (–)-carbovir and 3'-azido-3'-deoxythymidine against human immunodeficiency virus *in vitro*, *Antimicrob. Agents Chemother.*, 34, 1297–1300, 1990.
76. Exall, A.M., Jones, M.F., Mo, C.L., et al., Synthesis of (–)-aristeromycin and X-ray structure of (–)-carbovir, *J. Chem. Soc. Perkin Trans.*, 1, 2467–2477, 1991.
77. Pun, K.T., Baines, B.S., and Lawrence, G.C., Isolation, immobilization and use of adenosine deaminase in the synthesis of (–)-carbovir, a purine nucleoside analogue, 5th European congress on biotechnology, Copenhagen, Abs. no. TUP 111, 1990, p. 292.
78. Mahmoudian, M., Top 400 prescription drugs, *MedAd News*, 3–20, Eastman Chemical Company, USA, unpublished work. 2003.
79. Adamo, M.F.A., Aggarwal, V.K., and Sage, M.A., Epoxidation of alkenes by amine catalyst precursors: implication of aminium ion and radical cation intermediates, *J. Am. Chem. Soc.*, 122, 8317–8318, 2000.
80. Nieuwenhuijzen, J.W., Grimbergen, R.F.P., Koopman, C., et al., The role of nucleation inhibition in optical resolutions with families of resolving agents, *Angew. Chem. Int. Ed.*, 41, 4281–4286, 2002.
81. Henderson, K.W., Kerr, W.J., and Moir, J.H., Enantioselective deprotonation reactions using a novel homochiral magnesium amide base, *Chem. Commun.*, 479–480, 2000.
82. Balint, G., Egri, M., and Czugler, J., Resolution of 1-phenylethylamine by its acidic derivatives, *Tetrahedron Asymmetry*, 12, 1511–1518, 2001.
83. Varies, H., Wynberg, H., van Echten, E., et al., The family approach to the resolution of racemates, *Angew. Chem. Int. Ed.*, 37, 2349–2354, 1998.
84. Messina, F., Botta, M., Corelli, M.P., et al., Stereoselective synthesis of aryl-2-benzofuranmethanamines and aryl-1H-indole-2-methanamines through palladium-mediated annulation of chiral arylpropargylamines, *Tetrahedron Asymmetry*, 11, 1681–1685, 2000.
85. Luna, A., Alfonso, I., and Gotor, V., Biocatalytic approaches toward the synthesis of both enantiomers of *trans*-cyclopentane-1,2-diamine, *Org. Lett.*, 4, 3627–3629, 2002.
86. Takayama, S., Lee, S.T., Hung, S.C., et al., Designing enzymatic resolution of amines, *Chem. Commun.*, 127–128, 1999.
87. Gutman, A.L., Meyer, E., Kalerin, E., et al., Enzymatic resolution of racemic amines in continuous reactor in organic solvents, *Biotechnol. Bioeng.*, 40, 760–767, 1992.
88. Jaeger, K., Liebeton, K., Zonta, A., et al., Biotechnological application of *Pseudomonas aeruginosa* lipase: efficient kinetic resolution of amines and alcohols, *Appl. Microbiol. Biotechnol.*, 46, 99–105, 1996.
89. Stirling, D.I., The use of aminotransferases for the production of chiral amino acids and amines, in *Chirality in Industry*, Collins, A.N., et al., Eds., Wiley, New York, 1992, pp. 209–222.
90. Matcham, G., Bhatia, M., Lang, W., et al., Enzyme and reaction engineering in biocatalysis: synthesis of (*S*)-methoxyisopropylamine, *Chimia*, 53, 584–589, 1999.
91. Shin, J.S., Kim, B.G., Liese, A., et al., Kinetic resolution of chiral amines using enzyme membrane reactors, *Biotechnol. Bioeng.*, 73, 179–187, 2001.
92. Shin, J.S. and Kim, B.G., Comparison of the ω -transaminase from different microorganisms and application to production of chiral amines, *Biosci. Biotechnol. Biochem.*, 65, 1782–1788, 2001.
93. Takaaki, Y., Hiroshi, T., and Takeo Y., Production of optically active α -methylbenzylamine, Jap. Patent Appl. 4234993A, 1991.
94. Uematsu, N., Fujii, A., Hashiguchi, S., et al., Asymmetric transfer hydrogenation of imines, *J. Am. Chem. Soc.*, 118, 4916–4917, 1996.
95. Okuda, J., Verch, S., Sturmer, R., et al., Chiral complexes of titanium containing a linked amido cyclopentadienyl ligand: synthesis, structure, and asymmetric imine hydrogenation catalysis, *J. Organomet. Chem.*, 605, 55–67, 2000.

96. Kainz, S., Brinkmann, A., Leitner, W., et al., Iridium-catalyzed enantioselective hydrogenation of imines in supercritical carbon dioxide, *J. Am. Chem. Soc.*, 121, 6421–6429, 1999.
97. Hieber, G. and Ditrich, K., Introducing ChiPros biocatalytic production of chiral intermediates on a commercial scale, *Chimia Oggi*, 19, 16–20, 2001.
98. Reetz, M.T. and Schimossek, K., Lipase-catalyzed dynamic kinetic resolution of chiral amines: use of palladium as the racemisation catalyst, *Chimia*, 50, 668–689, 1996.
99. Choi, Y.K., Kim, M.J., Ahn, Y., et al., Lipase palladium-catalyzed asymmetric transformations of ketoximes to optically active amines, *Org. Lett.*, 3, 4099–4101, 2001.
100. Pamies, O., Ell, A.H., Samec, J.S.M., et al., An efficient and mild ruthenium-catalyzed racemisation of amines: application to the synthesis of enantiomerically pure amines, *Tetrahedron Lett.*, 43, 4699–4702, 2002.
101. Hirrlinger, B. and Stolz, A., Formation of a chiral hydroxamic acid with an amidase from *Rhodococcus erythropolis* MP50 and subsequent chemical Losen rearrangement to a chiral amine, *Appl. Environ. Microbiol.*, 63, 3390–3393, 1997.
102. Chapman, D.T., Crout, D.H.G., Mahmoudian, M., et al., Enantiomerically pure amines by a new method: biotransformation of oxalamic esters using the lipase from *Candida antarctica*, *Chem. Commun.*, 2415–2416, 1996.
103. Enright, A. and Mahmoudian, M., Deracemisation of chiral amines and amino acids, Glaxo Wellcome R&D, UK, unpublished work, 2001.
104. Alexeeva, M., Enright, A., Mahmoudian, M., et al., Deracemisation of amines, PCT Int. Appl. WO 030-80855, 2003.
105. Alexeeva, M., Enright, A., Mahmoudian, M., et al., Deracemisation of α -methylbenzylamine using an enzyme obtained by *in vivo* evolution, *Angew. Chem. Int. Ed.*, 41, 3177–3180, 2002.
106. Uppenberg, J., Hansen, M.T., Patkar, S., et al., The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*, *Structure*, 2, 293–308, 1994.
107. Chapman, D.T., Mahmoudian, M., and Crout, D.H.G., Glaxo Wellcome R&D, UK, unpublished work, 1995.
108. Hafner, E.W. and Wellner, D., Demonstration of imino acids as products of the reactions catalyzed by D- and L-amino acid oxidases, *Proc. Natl. Acad. Sci. USA*, 68, 987–991, 1971.
109. Huh, J.W., Yokoigawa, K., Soda, K.J., et al., Synthesis of L-proline from the racemate by coupling of enzymic enantiospecific oxidation and chemical non-enantiospecific reduction, *J. Ferment. Bioeng.*, 74, 189–190, 1992.
110. Huh, J.W., Yokoigawa, K., Soda, K.J., et al., Total conversion of racemic pipecolic acid into the L-enantiomer by a combination of enantiospecific oxidation with D-amino acid oxidase and reduction with sodium borohydride, *Biosci. Biotech. Biochem.*, 56, 2081–2082, 1992.
111. Quay, S. and Massey, V., Effect of pH on the interaction of benzoate and D-amino acid oxidase, *Biochemistry*, 16, 3348–3354, 1977.
112. Pollegioni, L., Buto, S., Fischer, W., et al., Characterization of D-amino acid oxidase from *Trigonopsis variabilis*, *Biochem. Mol. Biol. Int.*, 31, 709–717, 1993.
113. Pantaleone, D.P., Geller, A.M., and Taylor, P.P., Purification and characterization of an L-amino acid deaminase used to prepare unnatural amino acids, *J. Mol. Cat. B Enzyme*, 11, 795–803, 2001.
114. Braun, M., Kim, J.M., and Schmid, R.D., Purification and some properties of an extracellular L-amino acid oxidase from *Cellulomonas cellulans* AM8 isolated from soil, *Appl. Microbiol. Biotechnol.*, 37, 594–598, 1992.
115. Truong, M., Lecornue, F., and Fadel, A., First synthesis of (1*S*,2*S*)- and (1*R*,2*R*)-1-amino-2-isopropylcyclobutanecarboxylic acids by asymmetric Strecker reaction from 2-substituted cyclobutanones, *Tetrahedron Asymmetry*, 14, 1063–1072, 2003.
116. Johnstone, R.A.W., Wilby, A.H., and Entwistle, I.D., Heterogeneous catalytic transfer hydrogenation and its relation to other methods for reduction of organic compounds, *Chem. Rev.*, 85, 129–170, 1985.
117. Hutchins, R.O., Learn, K., Nazer, B., et al., Amine boranes as selective reducing and hydroborating agents: a review, *Org. Prep. Proc. Int.*, 16, 335–372, 1984.
118. Frebort, I., Matsushita, K., and Adachi, O., Involvement of multiple copper/topa containing and flavin containing amine oxidases and NADP aldehyde dehydrogenases in amine degradation by filamentous fungi, *J. Ferment. Bioeng.*, 84, 200–212, 1997.

119. Silverman, R.B., Cesarone, J.M., and Liu, X., Stereoselective ring opening of 1-phenylcyclopropylamine catalyzed by monoamine oxidase, *J. Am. Chem. Soc.*, 115, 4955–4961, 1993.
120. Schilling, B. and Lerch, K., Amine oxidases from *Aspergillus niger*: identification of a novel flavin-dependent enzyme, *Biochim. Biophys. Acta.*, 1243, 529–539, 1995.
121. Schilling, B. and Lerch, K., Cloning, sequencing and heterologous expression of the monoamine oxidase gene from *Aspergillus niger*, *Mol. Gen. Genet.*, 247, 430–438, 1995.
122. Sablin, S.O., Yankovskaya, V., Bernard, S., et al., Isolation and characterization of an evolutionary precursor of human monoamine oxidases A and B, *Eur. J. Biochem.*, 253, 270–279, 1998.
123. Korbel, G.A., Lalic, G., and Shair, M.D., Reaction microarrays: a method for rapidly determining the enantiomeric excess of thousands of samples, *J. Am. Chem. Soc.*, 123, 361–362, 2001.
124. Badalassi, F., Wahler, D., Klein, G., et al., A versatile periodate-coupled fluorogenic assay for hydrolytic enzymes, *Angew. Chem. Int. Ed.*, 39, 4067–4070, 2000.
125. Reetz, M.T. and Sostmann S., 2,15-Dihydroxyhexahelicene (HELIXOL): synthesis and use as an enantioselective fluorescent sensor, *Tetrahedron*, 57, 2515–2520, 2001.
126. Reetz, M.T., Becker, M.H., Klein, H.W., et al., A method for high-throughput screening of enantioselective catalysts, *Angew. Chem. Int. Ed.*, 38, 1758–1759, 1999.
127. Reetz, M.T., Kühling, K.M., Deege, A., et al., Super high throughput screening of enantioselective catalysts by using capillary array electrophoresis, *Angew. Chem. Int. Ed.*, 39, 3891–3893, 2000.
128. Ding, K., Ishii, A., and Mikami, K., Super high-throughput screening of chiral ligands and activators: asymmetric activation of chiral diol zinc catalysts by chiral nitrogen activators for the enantioselective addition of diethylzinc to aldehydes, *Angew. Chem. Int. Ed.*, 38, 497–501, 1999.
129. Taylor, S.J. and Morken, J.P., Thermographic selection of effective catalysts from an encoded polymer-bound library, *Science*, 280, 267–270, 1998.
130. Reetz, M.T., Becker, M.H., Kühling, K.M., et al., Time-resolved IR-thermographic detection and screening of enantioselectivity in catalytic reactions, *Angew. Chem. Int. Ed.*, 37, 2647–2649, 1998.
131. Reetz, M.T., Becker, M.H., Liebl, M., et al., IR-thermographic screening of thermoneutral or endothermic transformations: the ring closing olefin metathesis reaction, *Angew. Chem. Int. Ed.*, 39, 1236–1238, 2000.
132. Millot, N., Borman, P., Mahmoudian, M., et al., Rapid determination of enantiomeric excess using infrared thermography, *Org. Process R&D*, 6, 463–470, 2002.
133. Corey, E.J. and Helal, C.J., Reduction of carbonyl compounds with chiral oxazaborolidine catalysts: a new paradigm for enantioselective catalysis and a powerful new synthetic method, *Angew. Chem. Int. Ed.*, 37, 1986–2012, 1998.
134. Anderson, E.M., Larsson, K.M., and Kirk, O., One biocatalyst many applications: the use of *Candida antarctica* B lipase in organic synthesis, *Biocatal. Biotrans.*, 16, 181–185, 1998.

4 Biocatalysis for Synthesis for Chiral Pharmaceutical Intermediates

Ramesh N. Patel

CONTENTS

4.1	Introduction	105
4.2	Antiviral Drugs	105
4.2.1	Enzymatic Preparation of (1 <i>S</i> ,2 <i>S</i>)-[3-Chloro-2-Hydroxy-1-(Phenylmethyl)propyl]carbamic Acid, 1,1-Dimethylethyl Ester	105
4.2.2	Enzymatic Preparation of (1 <i>S</i> ,2 <i>R</i>)-[3-Chloro-2-Hydroxy-1-(Phenylmethyl)propyl]carbamic Acid, 1,1-Dimethylethyl Ester	106
4.2.3	Enzymatic Synthesis of (<i>S</i>)- <i>tert</i> -Leucine	107
4.2.4	Regioselective Enzymatic Aminoacylation	107
4.2.5	Crixivan (HIV-Protease Inhibitor)	108
4.2.5.1	Enzymatic Preparation of <i>cis</i> -(1 <i>S</i> ,2 <i>R</i>)-Indandiol and <i>trans</i> -(1 <i>R</i> ,2 <i>R</i>)-Indandiol	108
4.2.5.2	Enzymatic Preparation of (1 <i>S</i> ,2 <i>R</i>)-Indene Oxide	110
4.3	Anticancer Drugs	110
4.3.1	Paclitaxel Semisynthesis	110
4.4	Orally Active Taxane	114
4.4.1	Enzymatic Preparation of (3 <i>R-cis</i>)-3-Acetyloxy-4-(1,1-Dimethylethyl)-2-Azetidinone	114
4.5	Epothilones	115
4.5.1	Microbial Hydroxylation of Epothiolone B	115
4.6	Deoxyspergualin	116
4.6.1	Enantioselective Enzymatic Acetylation of Racemic 7-[<i>N,N'</i> -bis (Benzyloxycarbonyl)- <i>N</i> -(Guanidinoheptanoyl)]- α -Hydroxy-Glycine	116
4.7	Enzymatic Preparation of (<i>S</i>)-2-Chloro-1-(3-Chlorophenyl)ethanol	116
4.8	Antihypertensive Drugs	117
4.8.1	Angiotensin-Converting Enzyme Inhibitors	117
4.8.1.1	Captopril: Enzymatic Preparation of (<i>S</i>)-3-Acetylthio-2-Methylpropanoic Acid	117
4.8.1.2	Zofenopril: Enzymatic Preparation of (<i>S</i>)-3-Benzoylthio-2-Methylpropanoic Acid	118
4.8.1.3	Monopril: Enzymatic Preparation of (<i>S</i>)-2-Cyclohexyl- and (<i>S</i>)-2-Phenyl-1,3-Propanediol Monoacetates	119
4.8.1.4	Ceranopril: Enzymatic Preparation of <i>N</i> - ϵ -Carbobenzoxy-L-Oxylysine	120

4.8.2	Neutral Endopeptidase Inhibitors	120
4.8.2.1	Enzymatic Preparation of (<i>S</i>)- α -[(Acetylthio)methyl] benzenepropanoic Acid	120
4.8.3	Angiotensin-Converting Enzyme and Neutral Endopepsidase Inhibitors ...	120
4.8.3.1	Enzymatic Synthesis of (<i>S</i>)-6-Hydroxynorleucine	122
4.8.3.2	Enzymatic Synthesis of Allysine Ethylene Acetal	123
4.8.3.3	Enzymatic Synthesis of Thiazepine	124
4.9	Anticholesterol Drugs	124
4.9.1	Microbial Production of (<i>S</i>)-4-Chloro-3-Oxobutanoate Esters	124
4.9.2	Enzymatic Preparation of (<i>S</i>)-4-Chloro-3-Hydroxybutanoate	125
4.9.3	Enzymatic Preparation of (<i>R</i>)-4-Cyano-3-Hydroxybutyrate	125
4.9.4	Enzymatic Preparation of (<i>R</i>)- and (<i>S</i>)-Ethyl-3-Hydroxybutyrate	126
4.9.5	Enzymatic Synthesis of Ethyl (3 <i>R</i> ,5 <i>S</i>)-Dihydroxy-6-(Benzyloxy) Hexanoate	126
4.9.6	Enzymatic Preparation of a 2,4-Dideoxyhexose Derivative	127
4.9.7	Enzymatic Synthesis of <i>S</i> -[1-(Acetoxyl)-4-(3-Phenyl)butyl]phosphonic Acid, Diethyl Ester	128
4.10	Thromboxane A2 Antagonist	129
4.10.1	Enzymatic Preparation of Lactol [3 <i>aS</i> -(3 <i>a</i> α ,4 <i>a</i> α ,7 <i>a</i> α)]-4, 7-Epoxyisobenzofuran-1-(3 <i>H</i>)-ol	129
4.11	Calcium Channel Blocker	129
4.11.1	Enzymatic Preparation of [(3 <i>R</i> - <i>cis</i>)-1,3,4,5-Tetrahydro- 3-Hydroxy-4-(4-Methoxyphenyl)-6-(Trifluoromethyl)- 2 <i>H</i> -1-Benzazepin-2-one]	129
4.12	Potassium Channel Opener	131
4.12.1	Microbial Oxygenation to Prepare Chiral Epoxide and Diol	131
4.13	Melatonin Receptor Agonist	132
4.13.1	Enantioselective Enzymatic Hydrolysis of Racemic 1-{2',3'-Dihydro Benzo[b]furan-4'-yl}-1,2-Oxirane	132
4.13.2	Biocatalytic Dynamic Kinetic Resolution of (<i>R</i> , <i>S</i>)-1- {2',3'-Dihydrobenzo[b]furan-4'-yl}-Ethane-1,2-Diol	132
4.14	β -3-Receptor Agonist	133
4.14.1	Microbial Reduction of 4-Benzyloxy-3-Methanesulfonylamino- 2'-Bromoacetophenone	133
4.14.2	Enzymatic Resolution of Racemic α -Methylphenylalanine Amides	134
4.14.3	Enantioselective Hydrolysis of Diethyl Methyl-(4-Methoxyphenyl)-Propanedioate	135
4.15	β -2-Receptor Agonist	135
4.15.1	Enantioselective Enzymatic Acylation	135
4.16	Tryptase Inhibitor	136
4.16.1	Enzymatic Preparation of <i>S</i> - <i>N</i> (<i>tert</i> -Butoxycarbonyl)-3-Hydroxymethylpiperidine	136
4.17	Anti-Alzheimer's Drugs	137
4.17.1	Enzymatic Preparation of (<i>S</i>)-2-Pentanol and (<i>S</i>)-2-Heptanol	137
4.17.2	Enantioselective Enzymatic Reduction of 5-Oxohexanoate and 5-Oxohexanenitrile	138
4.17.3	Enantioselective Microbial Reduction of Substituted Acetophenone	138
4.18	Retinoid Receptor Gamma-Specific Agonists	139
4.18.1	Enzymatic Preparation of 2-(<i>R</i>)-Hydroxy-2-(1',2',3', 4'-Tetrahydro-1',1',4',4'-Tetramethyl-6'-Naphthalenyl) Acetate	139

4.19	Anti-Infective Drugs	141
4.19.1	Microbial Hydroxylation of Pleuromutilin or Mutilin	141
4.19.2	Enzymatic Preparation of (<i>R</i>)-1,3-Butanediol and (<i>R</i>)-4-Chloro-3-Hydroxybutanoate	141
4.19.3	Enzymatic Synthesis of L- β -Hydroxyvaline	142
4.20	α 1-Adrenoreceptor Antagonist	143
4.20.1	Enzymatic Esterification and Ammonolysis	143
4.21	Endothelin Receptor Antagonist	145
4.21.1	Enantioselective Microbial Reduction of Keto Ester and Chloroketone	145
4.22	Antianxiety Drug	146
4.22.1	Enzymatic Preparation of 6-Hydroxybuspirone	146
4.23	Antipsychotic Agent	147
4.23.1	Enzymatic Reduction of 1-(4-Fluorophenyl)-4-[4-(5-Fluoro- 2-Pyrimidinyl)-1-Piperazinyl]-1-Butanone	147
4.24	Enzymatic Acyloin Condensation	148
4.25	Enantioselective Enzymatic Cleavage of Carbobenzyloxy Groups	149
4.26	Conclusion	150
	Acknowledgment	150
	References	151

4.1 INTRODUCTION

Chirality is a key factor in the efficacy of many drug products and agrochemicals, and thus the production of single enantiomers of chiral intermediates has become increasingly important in the pharmaceutical industry [1]. Single enantiomers can be produced by chemical or chemo-enzymatic synthesis. The advantages of biocatalysis over chemical synthesis are that enzyme-catalyzed reactions are often highly enantioselective and regioselective. They can be carried out at ambient temperature and atmospheric pressure, thus avoiding the use of more extreme conditions that could cause problems with isomerization, racemization, epimerization, and rearrangement. Microbial cells and enzymes derived therefrom can be immobilized and reused for many cycles. In addition, enzymes can be overexpressed to make biocatalytic processes economically efficient, and enzymes with modified activity can be tailor-made. The preparation of thermostable and pH-stable enzymes by random and site-directed mutagenesis has led to the production of novel biocatalysts. A number of review articles [2–10] have been published on the use of enzymes in organic synthesis. This chapter provides examples of the use of enzymes for the synthesis of single enantiomers of key intermediates for drugs.

4.2 ANTIVIRAL DRUGS

4.2.1 ENZYMATIC PREPARATION OF (1*S*,2*S*)-[3-CHLORO-2-HYDROXY-1-(PHENYLMETHYL)PROPYL]CARBAMIC ACID, 1,1-DIMETHYLETHYL ESTER

An essential step in the life cycle of the human immunodeficiency virus (HIV-1) is the proteolytic processing of its precursor proteins by HIV-1 protease, a virally encoded enzyme. Inhibition of HIV-1 protease arrests the replication of HIV *in vitro*, and thus, HIV-1 protease is an attractive target for chemotherapeutic intervention. Barrish et al. [11] reported the discovery of a new class of selective HIV-protease inhibitors, which incorporate a C2 symmetric aminodiol core as its key structural feature. Members of this class, particularly

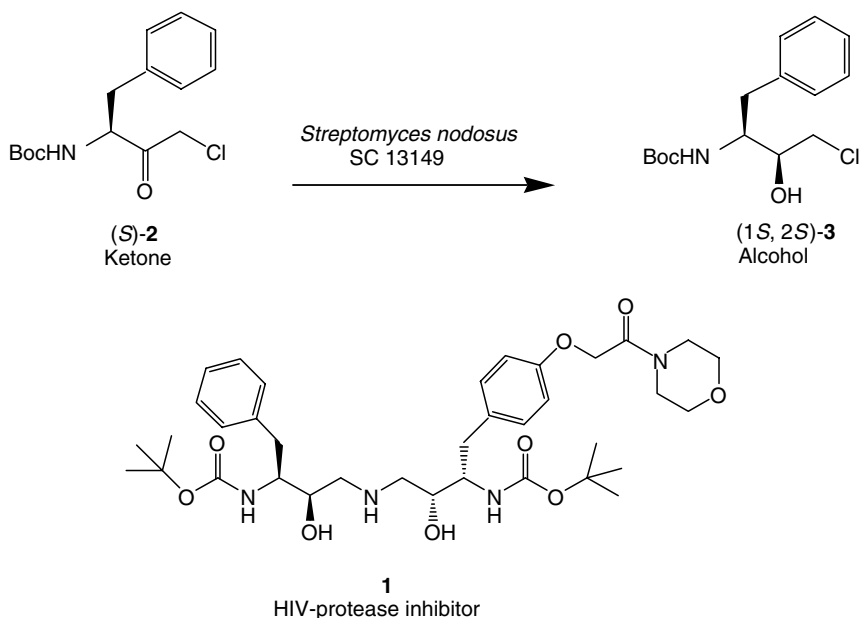


FIGURE 4.1 Synthesis of chiral intermediates for HIV-protease inhibitor **1**. Enantioselective enzymatic reduction of (1*S*)-[3-chloro-2-oxo-1(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester [(*S*)-**2**] to the corresponding (1*S*,2*S*)-**3** by *Streptomyces nodosus* SC 13149.

compound **1** (Figure 4.1), display potent anti-HIV activity in cell culture. The diastereoselective microbial reduction of (1*S*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester (**2**) to **3**, a key intermediate in the total chemical synthesis of compound **1**, has been demonstrated [12]. *Streptomyces nodosus* SC 13149 converted ketone **2** to the corresponding chiral alcohol **3** in a reaction yield of 67% with an enantiomeric excess (ee) of 99.9% and a diastereomeric purity of >99%. *Mortierella ramanniana* SC 13850 gave a reaction yield of 54% with an ee of 99.9% and a diastereomeric purity of 92% for **3**. A single-stage fermentation–biotransformation process was developed using cells of *S. nodosus* SC 13149. A reaction yield of 80% with a diastereomeric purity of >99% and an ee of 99.8% was obtained.

4.2.2 ENZYMATIC PREPARATION OF (1*S*,2*R*)-[3-CHLORO-2-HYDROXY-1-(PHENYLMETHYL)PROPYL]CARBAMIC ACID, 1,1-DIMETHYLETHYL ESTER

Atazanavir (**4**, Figure 4.2a) is an acyclic aza-peptidomimetic, a potent HIV-protease inhibitor [13,14]. An enzymatic process has been developed for the preparation of (1*S*,2*R*)-[3-chloro-2-hydroxy-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester (**5**) for the total synthesis of the HIV-protease inhibitor, atazanavir (Figure 4.2b). The diastereoselective reduction of (1*S*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester (**2**) was carried out using *Rhodococcus*, *Brevibacterium*, and *Hansenula* strains to provide **5**. Three strains of *Rhodococcus* gave >90% yield with a diastereomeric purity of >98% and an ee of 99.4% [15]. An efficient single-stage fermentation–biotransformation process was developed for the reduction of ketone **2** with cells of *Rhodococcus erythropolis* SC 13845 to yield **5** in 95% with a diastereomeric purity of 98.2% and an ee of 99.4%. Chemical reduction of chloroketone **2** using NaBH_4 produces primarily the undesired chlorohydrin diastereomer [16].

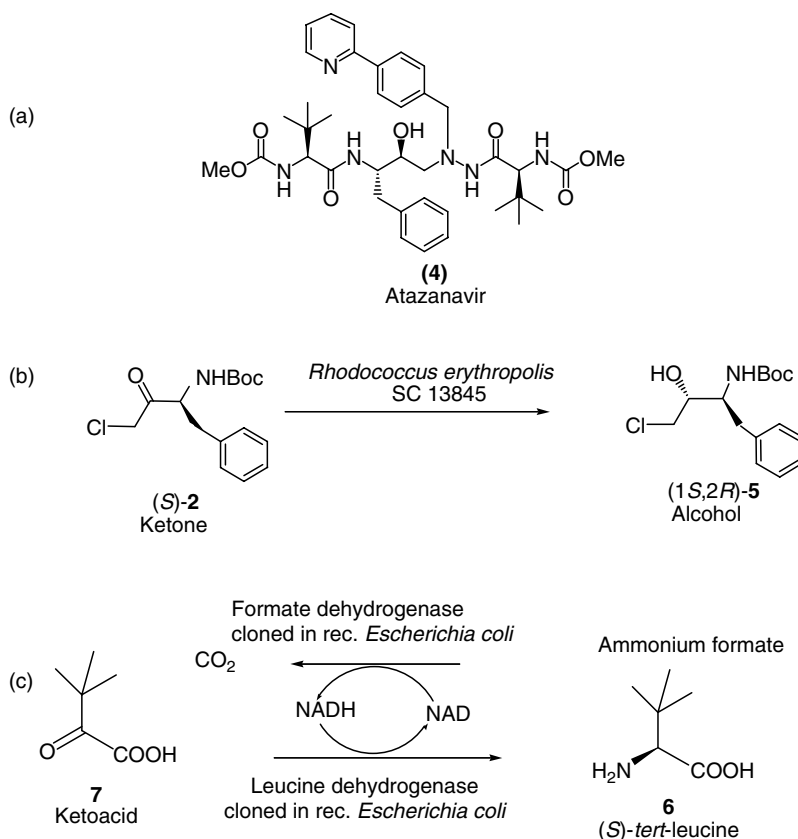


FIGURE 4.2 (a) Synthesis of chiral intermediates for antiviral agent, atazanavir **4**. (b) Enantioselective enzymatic reduction of (1S)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamate, 1,1-dimethylethyl ester (**2**) to the corresponding (1S,2R)-**5** by *Rhodococcus erythropolis* SC 13845. (c) Enzymatic reductive amination of ketoacid **7** to (S)-tert-leucine (**6**) by leucine dehydrogenase.

4.2.3 ENZYMATIC SYNTHESIS OF (S)-TERT-LEUCINE

Synthesis of atazanavir also required the (S)-tert-leucine **6** (Figure 4.2c). An enzymatic reductive amination of ketoacid **7** to amino acid **6** by recombinant *Escherichia coli* expressing leucine dehydrogenase from *Thermoactinomyces intermedius* was developed. The reaction required ammonia and NADH as a cofactor. Nicotinamide adenine dinucleotide (NAD) produced during the reaction was regenerated back to NADH using recombinant *E. coli* expressing formate dehydrogenase (FDH) from *Pichia pastoris*. A reaction yield of >95% with an ee of >99.5% was obtained for **6** at 100 g/L substrate input (R. Hanson, S. Goldberg, and R. Patel, unpublished results).

4.2.4 REGIOSELECTIVE ENZYMATIC AMINOACYLATION

Lobucavir (**12**, Figure 4.3) is a cyclobutyl guanine nucleoside analog under development as an antiviral agent for the treatment of herpes virus and hepatitis B [17]. A prodrug in which one of the two hydroxyls is coupled to valine, **14**, has also been considered for development. Regioselective aminoacylation is difficult to achieve by chemical procedures, but appeared to be suitable for an enzymatic approach [18]. Synthesis of the lobucavir L-valine prodrug **14**

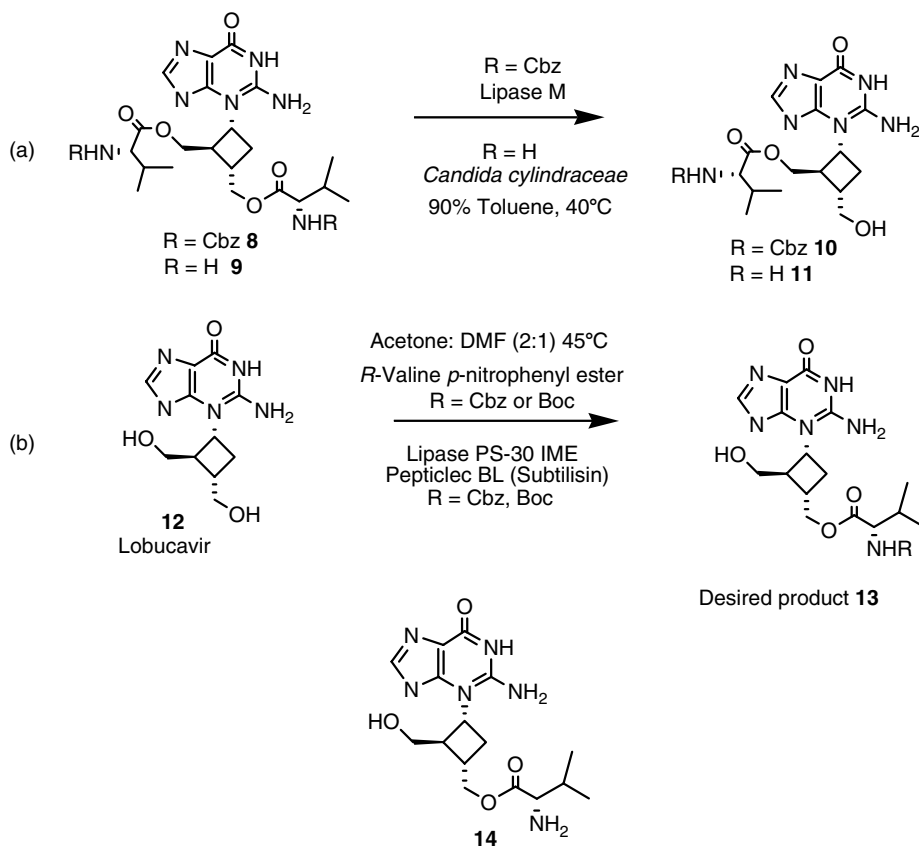


FIGURE 4.3 Synthesis of chiral intermediates for the lobucavir prodrug **14**. (a) Regioselective enzymatic hydrolysis of **8** and **9**. (b) Regioselective enzymatic aminoacylation of lobucavir (**12**).

requires regioselective coupling of one of the two hydroxyl groups of lobucavir (**12**) with valine. Enzymatic processes were developed for aminoacylation of either hydroxyl group of lobucavir [18]. The selective hydrolysis of the methyl ester **8** with lipase M gave **10** in 83% yield. When the methyl ester of **9** dihydrochloride was hydrolyzed with lipase from *Candida cylindraceae*, **11** was obtained in 87% yield. The final intermediates for lobucavir prodrug, the methyl ester of **13**, could be obtained by transesterification of lobucavir using ChiroCLEC BL (61% yield) or more selectively by using lipase from *Pseudomonas cepacia* (84% yield).

4.2.5 CRIXIVAN (HIV-PROTEASE INHIBITOR)

4.2.5.1 Enzymatic Preparation of *cis*-(1*S*,2*R*)-Indandiol and *trans*-(1*R*,2*R*)-Indandiol

Cis-(1*S*,2*R*)-indandiol **16** or *trans*-(1*R*,2*R*)-indandiol **16** (Figure 4.4a) are both potential precursors to *cis*-(1*S*,2*R*)-1-aminoindan-2-ol (**19**), a key chiral synthon for Crixivan (indinavir, **20**), a HIV-protease inhibitor. Enrichment and isolation of microbial cultures yielded two strains, *Rhodococcus* sp. B 264-1 (MB 5655) and I-24 (MA 7205), capable of biotransforming indene **15** to *cis*-(1*S*,2*R*)-indandiol and *trans*-(1*R*,2*R*)-indandiol, respectively [19]. Isolate MB 5655 was found to have a toluene dioxygenase, while isolate MA 7205 was found to harbor toluene and naphthalene dioxygenases as well as a naphthalene monooxygenase that catalyzes the above biotransformation. When scaled up in a 14 L fermentor, MB 5655 produced

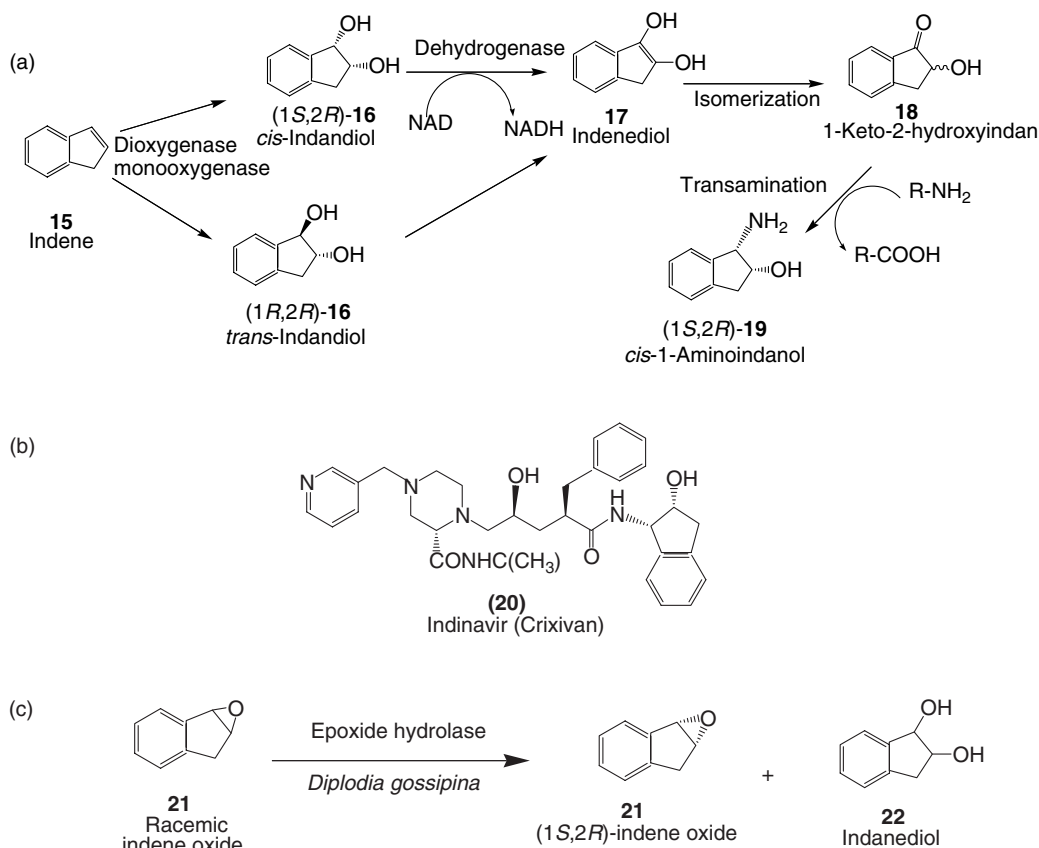


FIGURE 4.4 Synthesis of chiral intermediates for Crixivan (**20**). (a) Microbial oxygenation of indene **15** to *cis*-indandiol **16** and *trans*-indandiol **16**. (b) Resolution of racemic indene oxide **21** to (1*S*,2*R*)-indene oxide **21** by epoxide hydrolase from *Diplodia gossipina*.

up to 2.0 g/L of *cis*-(1*S*,2*R*)-indandiol **16** with an ee of >99%. *Rhodococcus* sp. MA 7205 cultivated under similar conditions produced up to 1.4 g/L of *trans*-(1*R*,2*R*)-indandiol **16** with an ee of >98%. Process development studies yielded titers of >4.0 g/L of *trans*-(1*R*,2*R*)-indandiol [20]. A metabolic engineering approach [21] and a directed evolution technique [22] were evaluated to avoid side reactions, block degradative pathways, and enhance the key reaction to convert indene to *cis*-aminoindanol **19** or *cis*-indanediol. The application of multiparameter flow cytometry was employed for the measurement of indene toxicity to the strain, and it was found that concentrations up to 0.25 g/L of indene (0.037 g indene/g dry cell weight) in batch bioconversions did not influence cell physiology. Using this information, the implementation of a single-phase indene fed-batch bioconversion was carried out. Cytoplasmic membrane integrity and membrane polarization of a large number of cells were measured during bioconversions and compared to a control in order to assess any toxic effects of indene feeding. The results indicated that indene supply at a rate of 0.1 g/L/h was feasible without any deleterious effects. *Cis*-(1*S*,2*R*)-indandiol **16** production rate was enhanced from 20 mg/L/h in a previously reported silicone oil two-liquid phase system up to 200 mg/L/h by a combination of suitable indene feeding rates in the stationary phase and operating with a high biomass concentration.

4.2.5.2 Enzymatic Preparation of (1*S*,2*R*)-Indene Oxide

In an alternate process, an epoxide hydrolase from *Diplodia gossypina* has been used for the resolution of racemic indene oxide **21** (Figure 4.4b). The desired enantiomer (1*S*,2*R*)-indene oxide (**21**), a chiral intermediate for Crixivan, was obtained in 14% yield and >99.9% ee. Indandiol **22** was obtained as a by-product [24].

4.3 ANTICANCER DRUGS

4.3.1 PACLITAXEL SEMISYNTHESIS

Among the antimitotic agents, paclitaxel (Taxol) (**23**, Figure 4.5), a complex, polycyclic diterpene, exhibits a unique mode of action on microtubule proteins responsible for the formation of the spindle during cell division. Paclitaxel is known to inhibit the depolymerization process of microtubulin [25]. Various types of cancers have been treated with paclitaxel, and the results in treatment of ovarian cancer and metastatic breast cancer are very promising. Paclitaxel was originally isolated from the bark of the yew *Taxus brevifolia* and has also been found in other *Taxus* spp. Paclitaxel was obtained from *T. brevifolia* bark in very low (0.07%) yield, and cumbersome purification from other related taxanes was required. It is estimated that about 20,000 pounds of yew bark (equivalent to about 3,000 trees) are needed to produce 1 kg of purified paclitaxel [27]. The development of a semisynthetic process for the production of paclitaxel from baccatin III (**24**) (paclitaxel without the C-13 side chain) or 10-deacetyl baccatin III (**25**) (10-DAB, paclitaxel without the C-13 side chain and the C-10 acetate) and C-13 paclitaxel side chain **27** or **31** was a very promising approach. Taxanes, baccatin III, and 10-DAB can be derived from renewable resources such as the needles, shoots, and young *Taxus* cultivars [28]. Thus, preparation of paclitaxel by a semisynthetic process would eliminate the cutting of yew trees.

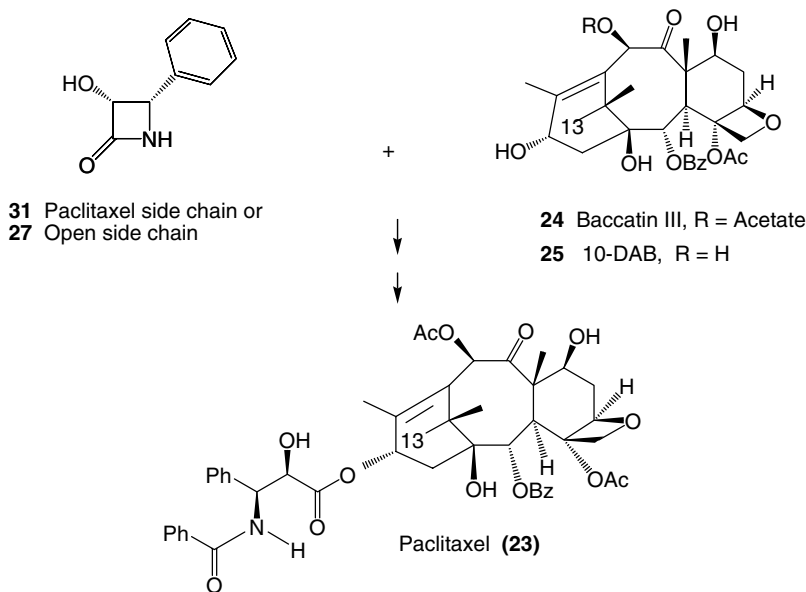


FIGURE 4.5 Semisynthesis of paclitaxel (**23**), an anticancer agent. Coupling of baccatin III (**24**) and C-13 paclitaxel side-chain synthons **27** or **31**.

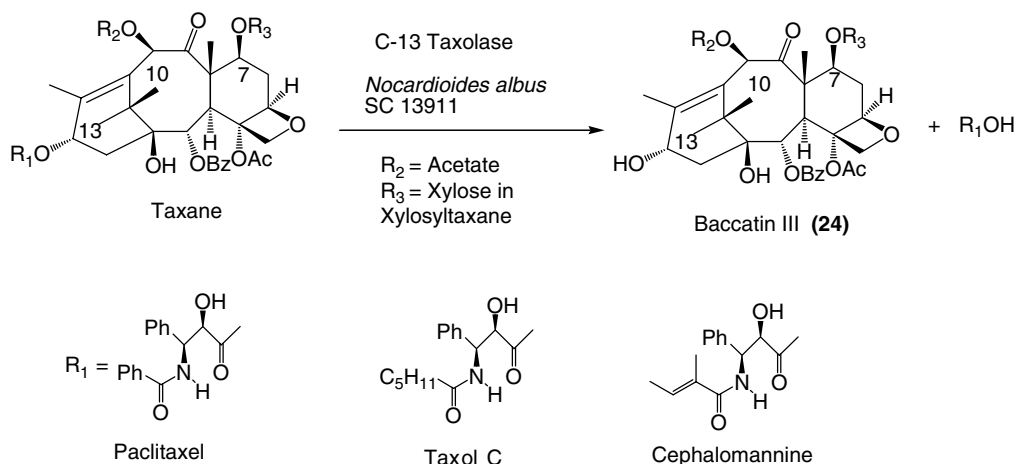


FIGURE 4.6 Enzymatic hydrolysis of C-13 side chain of taxanes by C-13 taxolase from *Nocardioides albus* SC 13911.

Using selective enrichment techniques, two strains of *Nocardioides* were isolated from soil samples that contained novel enzymes C-13 taxolase and C-10 deacetylase [29,30]. The extra-cellular C-13 taxolase derived from the filtrate of the fermentation broth of *Nocardioides albus* SC 13911 catalyzed the cleavage of the C-13 side chain from paclitaxel and related taxanes such as taxol C, cephalomannine, 7- β -xylosyltaxol, 7- β -xylosyl-10-deacetyltaxol, and 10-deacetyltaxol (Figure 4.6). The intracellular C-10 deacetylase derived from fermentation of *Nocardioides luteus* SC 13912 catalyzed the cleavage of the C-10 acetate from paclitaxel, related taxanes, and baccatin III to yield 10-DAB (Figure 4.7). The C-7 xylosidase derived from fermentation of *Moraxella* sp. (Figure 4.8) catalyzed the cleavage of the C-7 xylosyl group [31] from various taxanes. Fermentation processes were developed for growth of *N. albus* SC

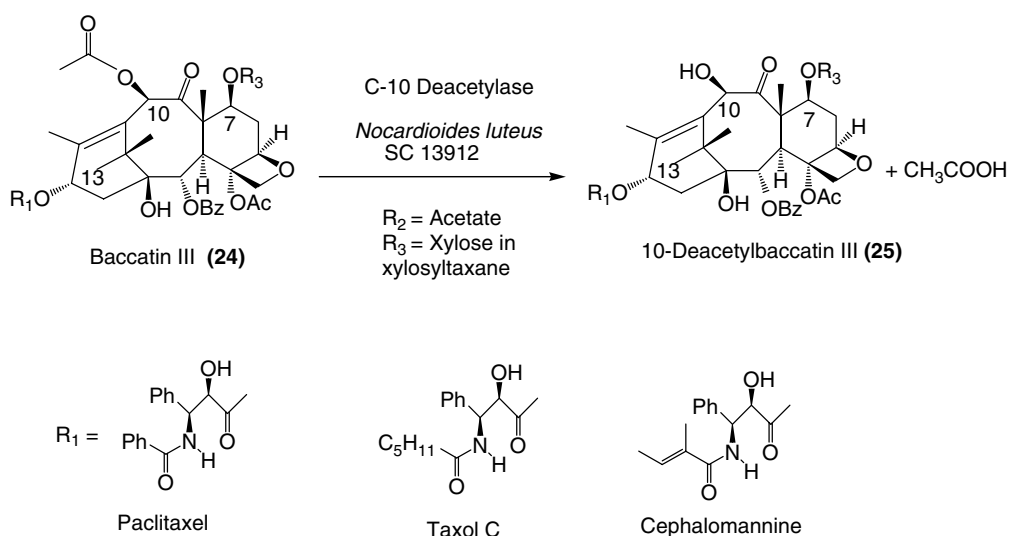


FIGURE 4.7 Enzymatic hydrolysis of C-10 acetate of taxanes and baccatin III **24** by C-10 deacetylase from *Nocardioides luteus* SC 13912.

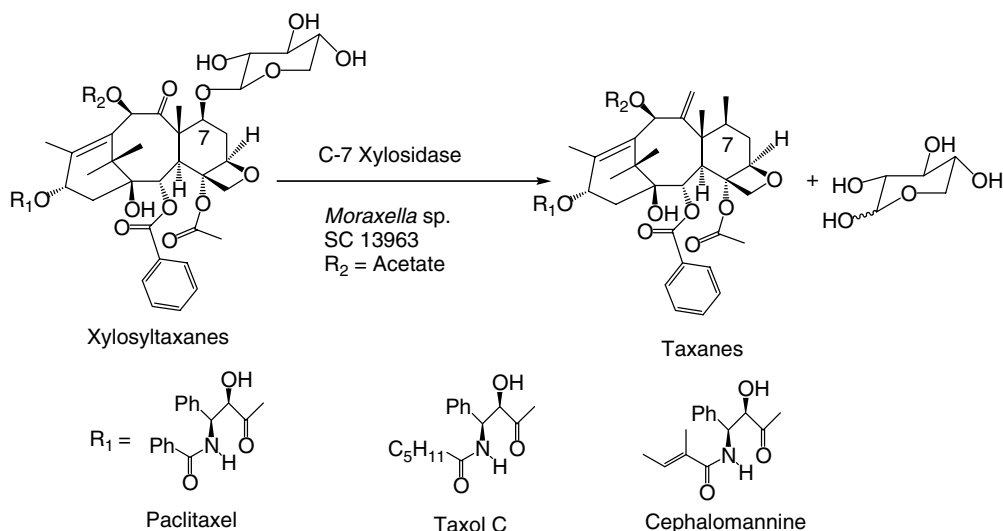


FIGURE 4.8 Enzymatic hydrolysis of C-7 xylose by C-7 xylosidase from *Moraxella* sp. 13963.

13911 and *N. luteus* SC 13912 to produce C-13 taxolase and C-10 deacetylase, respectively, in 5000 L batches, and a bioconversion process was demonstrated for the conversion of paclitaxel, and related taxanes in extracts of *Taxus* cultivars to the single compound 10-DAB **25** using both enzymes. In the bioconversion process, ethanolic extracts of the whole young plant of five different cultivars of *Taxus* were first treated with a crude preparation of the C-13 taxolase to give complete conversion of measured taxanes to baccatin III **24** and 10-DAB **25** in 6 h. *N. luteus* SC 13192 whole cells were then added to the reaction mixture to give complete conversion of baccatin III **24** to 10-DAB **25**. The concentration of 10-DAB **25** was increased by 5.5 to 24-fold in the extracts from various *Taxus* cultivars by treatment with the two enzymes. The bioconversion process was also applied to extracts of the bark of *T. braviifolia* to give a 12-fold increase in 10-DAB **25** concentration. Enhancement of the 10-DAB concentration in yew extracts was potentially useful in increasing the amount and purification of this key precursor for the paclitaxel semisynthetic process using renewable resources.

Another key precursor for the paclitaxel semisynthetic process is the chiral C-13 paclitaxel side chain. Two different enantioselective enzymatic processes were developed for the preparation of the chiral C-13 paclitaxel side-chain synthon [32,33]. In one process, the enantioselective microbial reduction of 2-keto-3-(*N*-benzoylamino)-3-phenyl propionic acid ethyl ester (**26**) (Figure 4.9) to yield (2*R*,3*S*)-*N*-benzoyl-3-phenyl isoserine ethyl ester (**27**) has been demonstrated using two strains of *Hansenula* [32]. Preparative-scale bioreduction of ketone **26** was demonstrated using cell suspensions of *Hansenula polymorpha* SC 13865 and *Hansenula fabianii* SC 13894 in independent experiments. In both batches, reaction yields of >80% and ees of >94% were obtained for **27**. A 20% yield of the undesired antiastereomers was obtained with *H. polymorpha* SC 13865 compared with a 10% yield with *H. fabianii* SC 13894. A 99% ee was obtained with *H. polymorpha* SC 13865 compared with a 94% ee with *H. fabianii* SC 13894. In a single-stage bioreduction process, cells of *H. fabianii* were grown in a 15 L fermentor for 48 h; then the bioreduction process was initiated by addition of 30 g of substrate and 250 g of glucose and continued for 72 h. A reaction yield of 88% with an ee of 95% was obtained for **27**.

In an alternate process for the preparation of the C-13 paclitaxel side chain, the enantioselective enzymatic hydrolysis of racemic acetate *cis*-3-(acetyloxy)-4-phenyl-2-azetidinone (**28**)

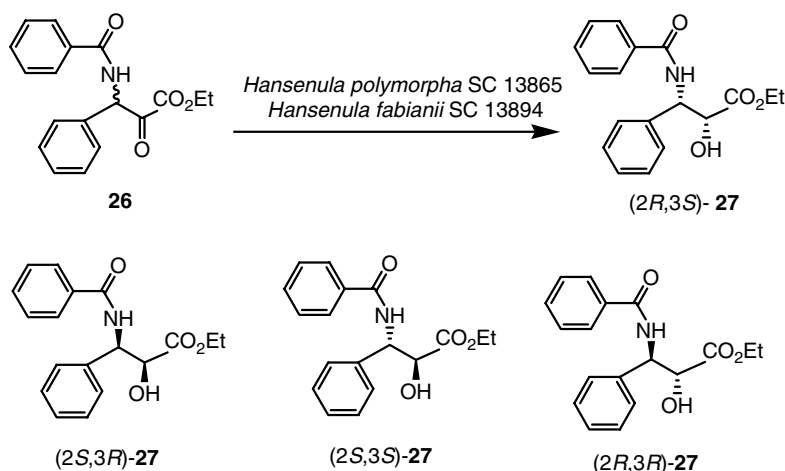


FIGURE 4.9 Enzymatic synthesis of C-13 side chain of paclitaxel **23**: enantioselective microbial reduction of 2-keto-3-(*N*-benzoylamino)-3-phenyl propionic acid, ethyl ester (**26**).

(Figure 4.10) to the corresponding (*S*)-alcohol **29** and the unreacted desired (*R*)-acetate **30** was demonstrated [33] using lipase PS-30 from *P. cepacia* (Amano International Enzyme Company) and BMS lipase (extracellular lipase derived from the fermentation of *Pseudomonas* sp. SC 13856). Reaction yields of >48% (theoretical maximum yield 50%) with ees of >99.5% were obtained for the (*R*)-acetate. BMS lipase and lipase PS-30 were immobilized on Accurel polypropylene (PP), and the immobilized lipases were reused (ten cycles) without loss of enzyme activity, productivity, or the ee of the product **30** in the resolution process. The enzymatic process was scaled up to 250 L (2.5 kg substrate input) using immobilized BMS lipase and lipase PS-30, respectively. From each reaction batch, *R*-acetate **30** was isolated in 45% yield (theoretical maximum yield 50%) and 99.5% ee. The (*R*)-acetate was chemically converted to (*R*)-alcohol **31**. The C-13 paclitaxel side-chain synthon (*2R,3S*-**27** or **31**)

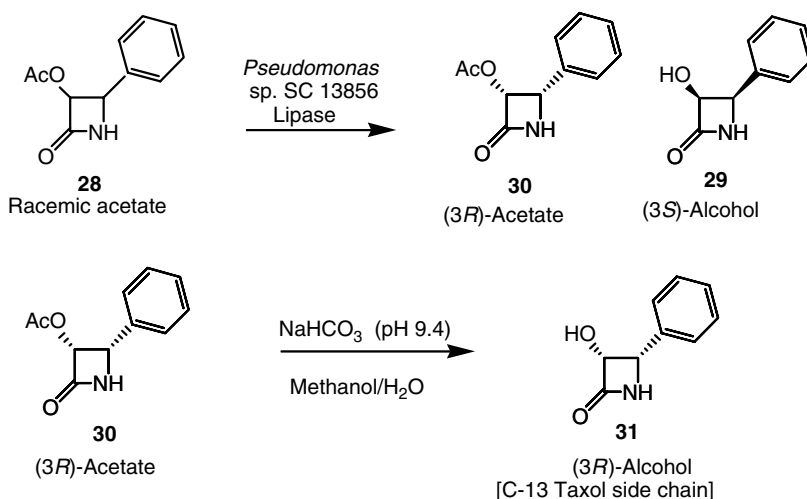


FIGURE 4.10 Enantioselective enzymatic hydrolysis of *cis*-3-(acetyloxy)-4-phenyl-2-azetidinone **28**.

produced either by the reductive or resolution process could be coupled to bacattin III (**24**) after protection and deprotection to prepare paclitaxel by a semisynthetic process [28].

4.4 ORALLY ACTIVE TAXANE

4.4.1 ENZYMATIC PREPARATION OF (3*R*-*cis*)-3-ACETYLOXY-4-(1,1-DIMETHYLETHYL)-2-AZETIDINONE

Due to the poor solubility of paclitaxel, various groups are involved in the development of water-soluble taxane analogs [34–36]. Taxane **32** (Figure 4.11a) is a water-soluble taxane derivative which, when given orally, was as effective as i.v. paclitaxel in five tumor models [murine M109 lung and C3H mammary 16/C cancer, human A2780 ovarian cancer cells (grown in mice and rats) and HCT/pk colon cancer] [35].

The chiral intermediate (3*R*-*cis*)-3-acetyloxy-4-(1,1-dimethylethyl)-2-azetidinone (**35**, Figure 4.11b) was prepared for the semisynthesis of the new taxane **32**. The enantioselective enzymatic hydrolysis of *cis*-3-acetyloxy-4-(1,1-dimethylethyl)-2-azetidinone (**33**) to the

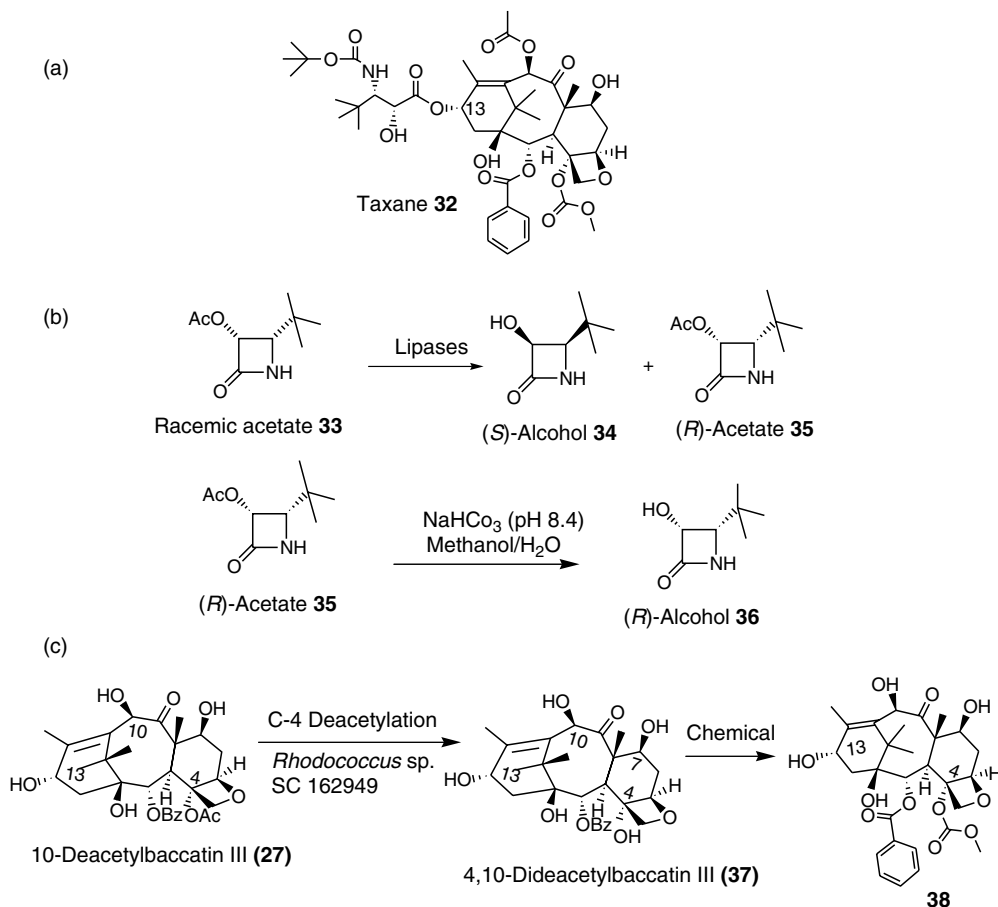


FIGURE 4.11 (a) Enzymatic synthesis of C-13 side chain **36** of orally active taxane **32**. (b) Enantioselective enzymatic hydrolysis of *cis*-3-acetyloxy-4-(1,1-dimethylethyl)-2 azetidinone (**33**). (c) Enzymatic C-4 deacetylation of 10-deacetylbaccatin III (**27**).

hydroxylation process was developed for conversion of epothilone B **39** to epothilone F **40** by *Amycolatopsis orientalis* SC 15847 [47].

4.6 DEOXYSPERGUALIN

4.6.1 ENANTIOSELECTIVE ENZYMATIC ACETYLATION OF RACEMIC

7-[*N,N'*-bis(BENZYLOXYCARBONYL)-*N*-(GUANIDINOHEPTANOYL)]- α -HYDROXY-GLYCINE

An antitumor antibiotic spergualin was discovered in the culture filtrate of a bacterial strain and its structure was determined to be 15*S*-1-amino-10-guanidino-11,15-dihydroxy-4,9,12-triazanonadecane-10,13-dione [48]. The total synthesis was accomplished by the acid-catalyzed condensation of 11-amino-1,1-dihydroxy-3,8-diazaundecane-2-one with (*S*)-7-guanidino-3-hydroxy-heptanamide followed by the separation of the 11-epimeric mixture. Antibacterial or antitumor activity of the racemic spergualin was about half of that of the natural spergualin [49] indicating the importance of the configuration at C-11 for antitumor activity.

The lipase-catalyzed enantioselective acetylation of racemic 7-[*N,N'*-bis(benzyloxycarbonyl)-*N*-(guanidinoheptanoyl)]- α -hydroxy-glycine (**41**, Figure 4.13) to the corresponding (*S*)-acetate **42** and unreacted alcohol (*R*)-**43** has been developed [50]. (*S*)-acetate **42** is a key intermediate in the chemical synthesis of (–)-15-deoxyspergualin (**44**), a related immunosuppressive agent and antitumor antibiotic [51]. The reaction was carried out in methyl ethyl ketone (MEK) using lipase from *Pseudomonas* sp. (lipase AK) with vinyl acetate as the acylating agent. A reaction yield of 48% (theoretical maximum yield 50%) with an ee of 98% was obtained for (*S*)-acetate **42**. The unreacted alcohol (*R*)-**43** was obtained in 41% yield and 94% ee.

4.7 ENZYMATIC PREPARATION OF (*S*)-2-CHLORO-1-(3-CHLOROPHENYL)ETHANOL

The synthesis of the leading candidate compound in an anticancer program [52,53] required (*S*)-2-chloro-1-(3-chlorophenyl)ethanol (**45**, Figure 4.14) as an intermediate. Other possible candidate compounds used analogs of the (*S*)-alcohol. About 100 microbial cultures were screened for reduction of the corresponding ketone **46** to the (*S*)-alcohol **45**, and *H. polymorpha* SC 13824 (73.8% ee) and *R. globerulus* SC SC16305 (71.8% ee) had the highest

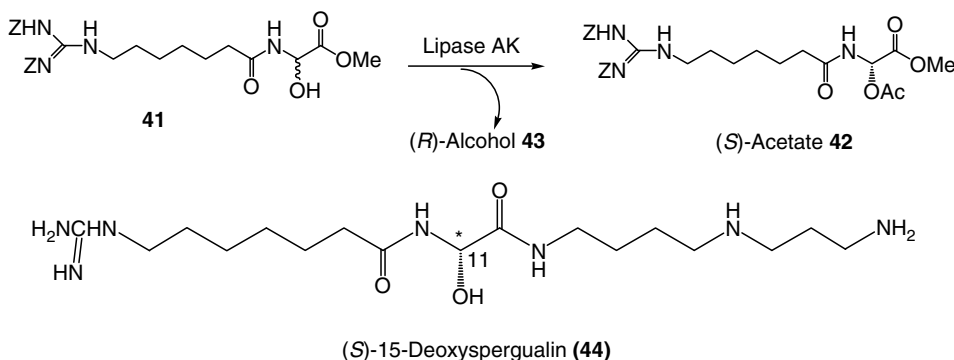


FIGURE 4.13 Synthesis of chiral intermediates for antitumor antibiotic 15-deoxyspergualin (**44**): enantioselective enzymatic acylation of racemic **41** to yield (*S*)-acetate **42**.

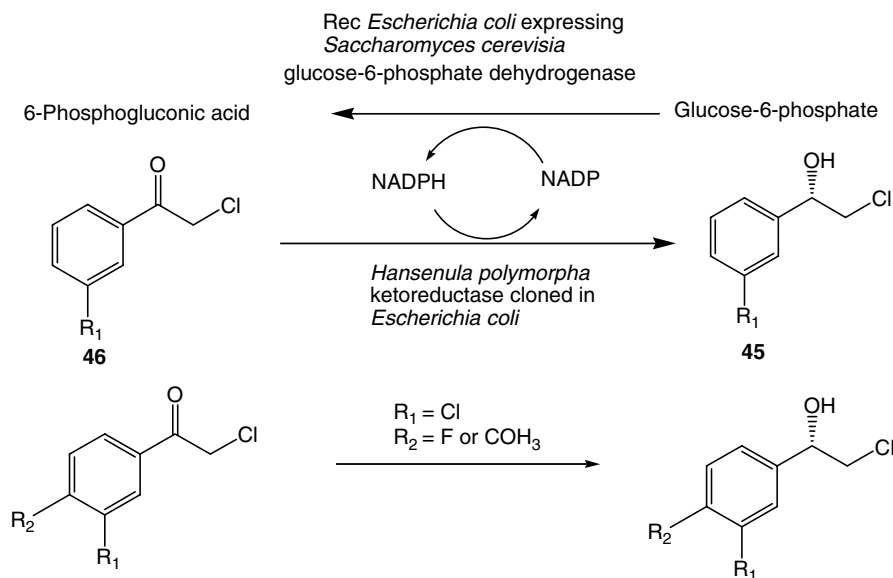


FIGURE 4.14 Enantioselective enzymatic reduction of **46** to **45**, an intermediate in anticancer drug.

enantioselectivity. A ketoreductase from *H. polymorpha*, after purification to homogeneity, gave (*S*)-alcohol **45** with 100% ee. Amino acid sequences from the purified enzyme were used to design PCR primers for cloning the ketoreductase. The cloned ketoreductase required NADP(H), had a subunit molecular weight of 29,220, and a native molecular weight of 88,000. The cloned ketoreductase was expressed in *E. coli* together with a cloned glucose-6-phosphate dehydrogenase from *Saccharomyces cerevisiae* to allow regeneration of the NADPH required by the ketoreductase. An extract of *E. coli* containing the two recombinant enzymes was used to reduce 2-chloro-1-(3-chloro-4-fluorophenyl)-ethanone (**46**) and two related ketones to the corresponding (*S*)-alcohols. Intact *E. coli* cells provided with glucose were used to prepare (*S*)-**45** in 89% yield with 100% ee [54].

4.8 ANTIHYPERTENSIVE DRUGS

4.8.1 ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

4.8.1.1 Captopril: Enzymatic Preparation of (*S*)-3-Acetylthio-2-Methylpropanoic Acid

Captopril is designated chemically as 1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline (**47**, Figure 4.15). It is used as an antihypertensive agent through suppression of the renin-angiotensin-aldosterone system [55,56]. Captopril prevents the conversion of angiotensin I to angiotensin II (AII) by inhibition of angiotensin-converting enzyme (ACE). The potency of captopril **47** as an inhibitor of ACE depends critically on the configuration of the mercaptoalkanoyl moiety; the compound with the *S*-configuration is about 100 times more active than its corresponding *R*-isomer [57]. The required 3-mercapto-(2*S*)-methylpropionic acid moiety has been prepared from the microbially derived chiral 3-hydroxy-(2*R*)-methylpropionic acid, which is obtained by the hydroxylation of isobutyric acid [58]. The synthesis of the (*S*)-side chain of captopril by the lipase-catalyzed enantioselective hydrolysis of the thioester bond of racemic 3-acetylthio-2-methylpropanoic acid (**48**) to yield (*S*)-**49** has been demonstrated [59]. Among various lipases evaluated, the lipase from *Rhizopus oryzae* ATCC 24563

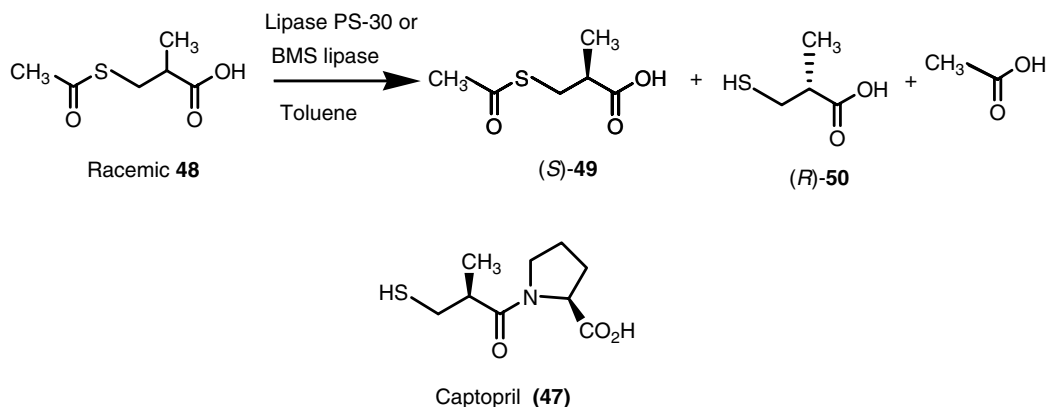


FIGURE 4.15 Enzymatic synthesis of captopril (**47**) side-chain synthon: enantioselective enzymatic hydrolysis of racemic 3-acylthio-2-methylpropanoic acid (**48**).

(heat-dried cells) and lipase PS-30 from *P. cepacia* in organic solvent systems (1,1,2-trichloro-1,2,2-trifluoroethane or toluene) catalyzed the hydrolysis of the thioester bond of the undesired enantiomer of racemic **48** to yield desired (*S*)-**49**, (*R*)-3-mercapto-2-methylpropanoic acid (**50**), and acetic acid (Figure 4.15). Reaction yields of >24% (maximum theoretical yield is 50%) with ees of >95% were obtained for (*S*)-**49** using each lipase.

4.8.1.2 Zofenopril: Enzymatic Preparation of (*S*)-3-Benzoylthio-2-Methylpropanoic Acid

In an alternative approach to prepare the chiral side chain of captopril (**47**), and zofenopril (**51**), the lipase-catalyzed enantioselective esterification of racemic 3-benzoylthio-2-methylpropanoic acid (**52**, Figure 4.16) in an organic solvent was demonstrated to yield *R*-(+) methyl ester **53** and unreacted acid enriched in the desired (*S*)-**54** [60]. Using lipase PS-30 with toluene as solvent and methanol as nucleophile, the desired (*S*)-**54** was obtained in 37% yield (maximum theoretical yield is 50%) with 97% ee. The amount of water and the concentration of methanol supplied in the reaction mixture were very critical. Water was used at 0.1% concentration in the reaction mixture. Higher than 1% water led to the

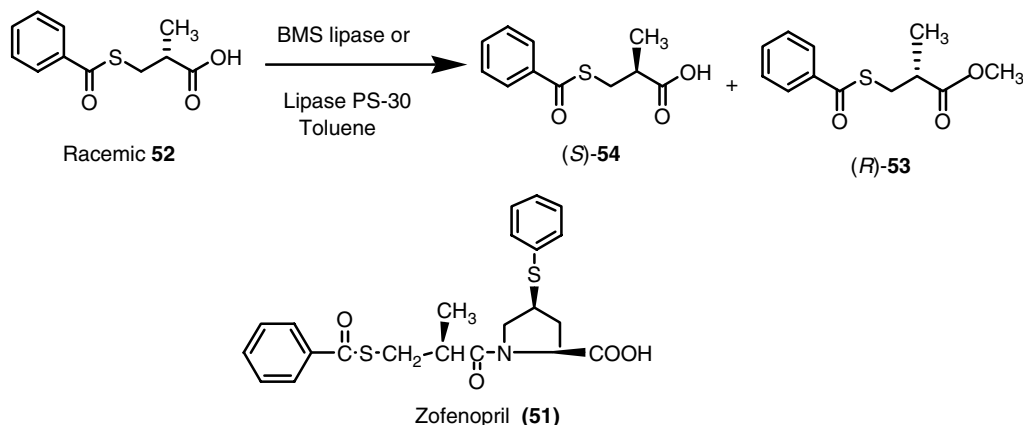


FIGURE 4.16 Synthesis of zofenopril (**51**) side-chain (*S*)-**54**: enantioselective enzymatic esterification of racemic 3-benzoylthio-2-methylpropanoic acid (**52**).

aggregation of enzyme in the organic solvent with a decrease in the rate of reaction due to mass transfer limitation. The rate of esterification decreased as the methanol/substrate ratio was increased from 1:1 to 4:1. Higher methanol concentrations probably inhibited the esterification reaction by stripping the essential water from the enzyme. Crude lipase PS-30 was immobilized on Accurel polypropylene (PP) in absorption efficiencies of 98.5%. The immobilized lipase efficiently catalyzed the esterification reaction, giving 45% reaction yield with 97.7% ee of (*S*)-**54**. The immobilized enzyme under identical conditions gave a similar ee and yield of product in 23 additional reaction cycles without any loss of activity and productivity. (*S*)-**54** is a key chiral intermediate for the synthesis of captopril [61] or zofenopril [62].

4.8.1.3 Monopril: Enzymatic Preparation of (*S*)-2-Cyclohexyl- and (*S*)-2-Phenyl-1,3-Propanediol Monoacetates

(*S*)-2-Cyclohexyl-1,3-propanediol monoacetate (**55**) and (*S*)-2-phenyl-1,3-propanediol monoacetate (**56**) are key chiral intermediates for the chemo-enzymatic synthesis of Monopril (**57**, Figure 4.17), an antihypertensive drug that acts as an ACE inhibitor. The asymmetric hydrolysis of 2-cyclohexyl-1,3-propanediol diacetate (**58**) and 2-phenyl-1,3-propanediol diacetate (**59**) to the corresponding (*S*)-monoacetate **55** and (*S*)-monoacetate **56** by porcine pancreatic lipase (PPL) and *Chromobacterium viscosum* lipase has been demonstrated [63]. In a biphasic system using 10% toluene, reaction yields of >65% with ees of 99% were obtained for (*S*)-**55** using each enzyme. (*S*)-**56** was obtained in 90% reaction yield with 99.8% ee using *C. viscosum* lipase under similar conditions.

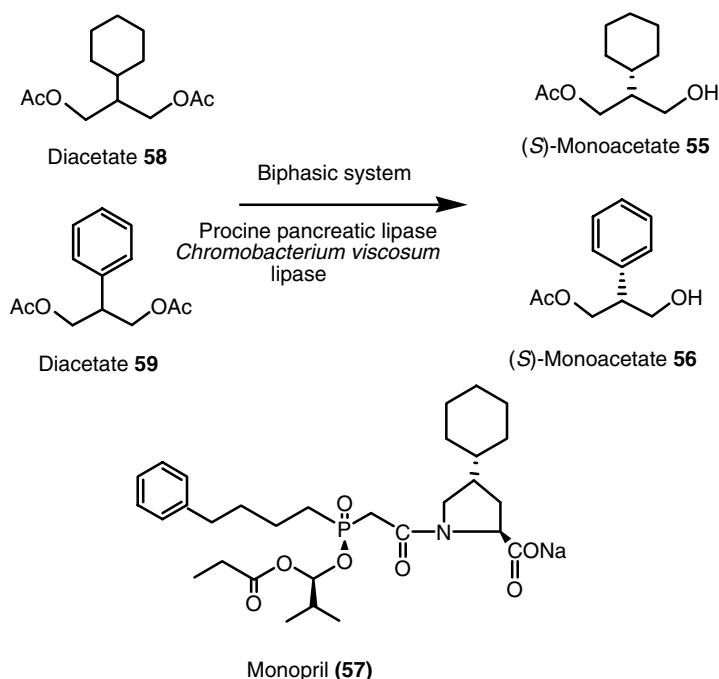


FIGURE 4.17 Preparation of chiral synthon for monopril (**57**): asymmetric enzymatic hydrolysis of 2-cyclohexyl-**58** and 2-phenyl-1,3-propanediol diacetate (**59**) to the corresponding (*S*)-monoacetates **55** and **56**.

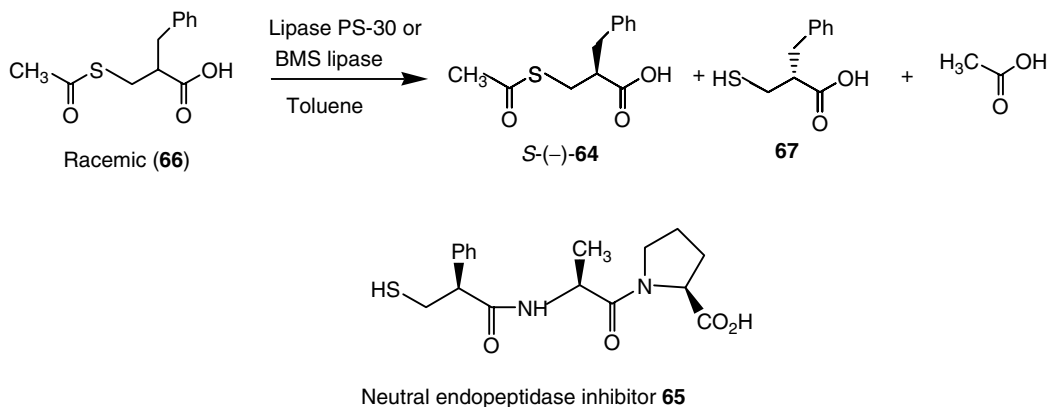


FIGURE 4.19 Preparation of chiral synthon for neutral endopeptidase inhibitor **65**: enantioselective enzymatic hydrolysis of racemic α -[(acetylthio)methyl]benzenepropanoic acid (**66**).

28-amino acid peptide secreted by the heart in response to atrial distension. By interaction with its receptor, ANP promotes the generation of cyclic guanosine monophosphate (cGMP) through guanylate cyclase activation, thus resulting in vasodilatation, natriuresis, diuresis, and inhibition of aldosterone. Therefore, simultaneous potentiation of ANP by NEP inhibition and attenuation of AII by ACE inhibition should lead to complementary effects in the management of hypertension and congestive heart failure [68].

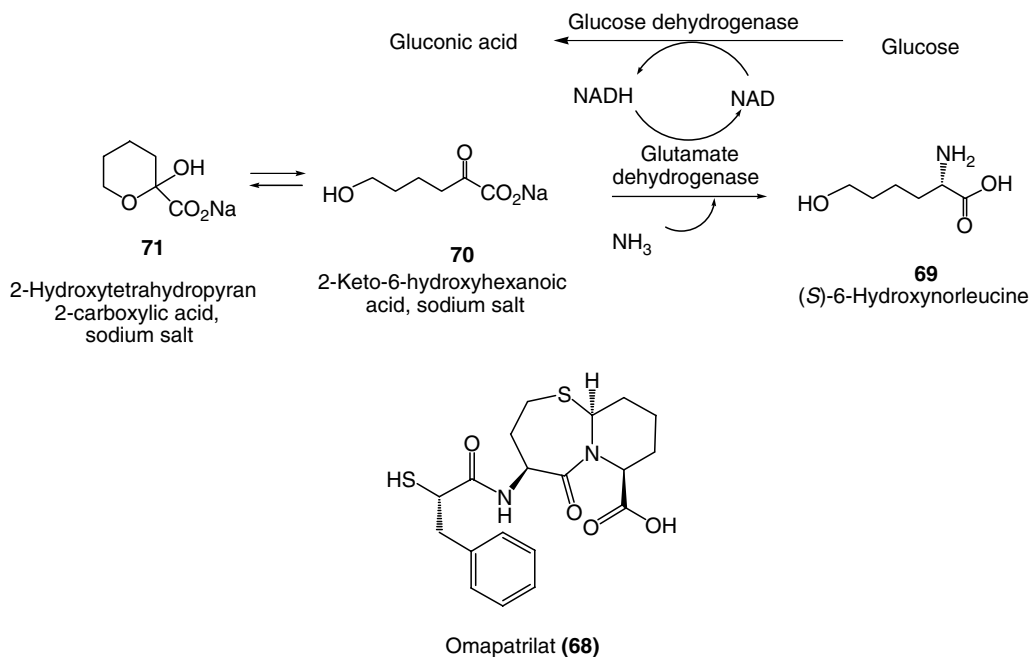


FIGURE 4.20 Enzymatic synthesis of chiral synthon for Omapatrilat (**68**): reductive amination of sodium 2-keto-6-hydroxyhexanoic acid (**70**) to (S)-6-hydroxynorleucine (**69**) by glutamate dehydrogenase.

4.8.3.1 Enzymatic Synthesis of (*S*)-6-Hydroxynorleucine

(*S*)-6-Hydroxynorleucine (**69**, Figure 4.20) is a key intermediate in the synthesis of omapatrilat. Reductive amination of ketoacids using amino acid dehydrogenases has been a useful method for the synthesis of natural and unnatural amino acids [69,70]. The synthesis and complete conversion of 2-keto-6-hydroxyhexanoic acid (**70**) to (*S*)-6-hydroxynorleucine (**69**) was demonstrated by reductive amination using phenylalanine dehydrogenase (PDH) from *Sporosarcina* sp. or beef liver glutamate dehydrogenase [71]. Beef liver glutamate dehydrogenase was used for preparative reactions at 100 g/L substrate concentration. As depicted, compound **70**, in equilibrium with 2-hydroxytetrahydropyran-2-carboxylic acid sodium salt (**71**), was converted to **69**. The reaction requires ammonia and NADH. NADH produced during the reaction was recycled to NAD by the oxidation of glucose to gluconic acid using glucose dehydrogenase from *Bacillus megaterium*. The reaction was complete in about 3 h, with reaction yields of 92% and ees of >99% for (*S*)-6-hydroxynorleucine.

The synthesis and isolation of ketoacid **70** required several steps. In a second, more convenient, process the ketoacid was prepared by treatment of racemic 6-hydroxynorleucine (**72**) (produced by hydrolysis of 5-(4-hydroxybutyl) hydantoin (**73**)) with D-amino acid oxidase and catalase (Figure 4.21). After the ee of the remaining (*S*)-6-hydroxynorleucine had

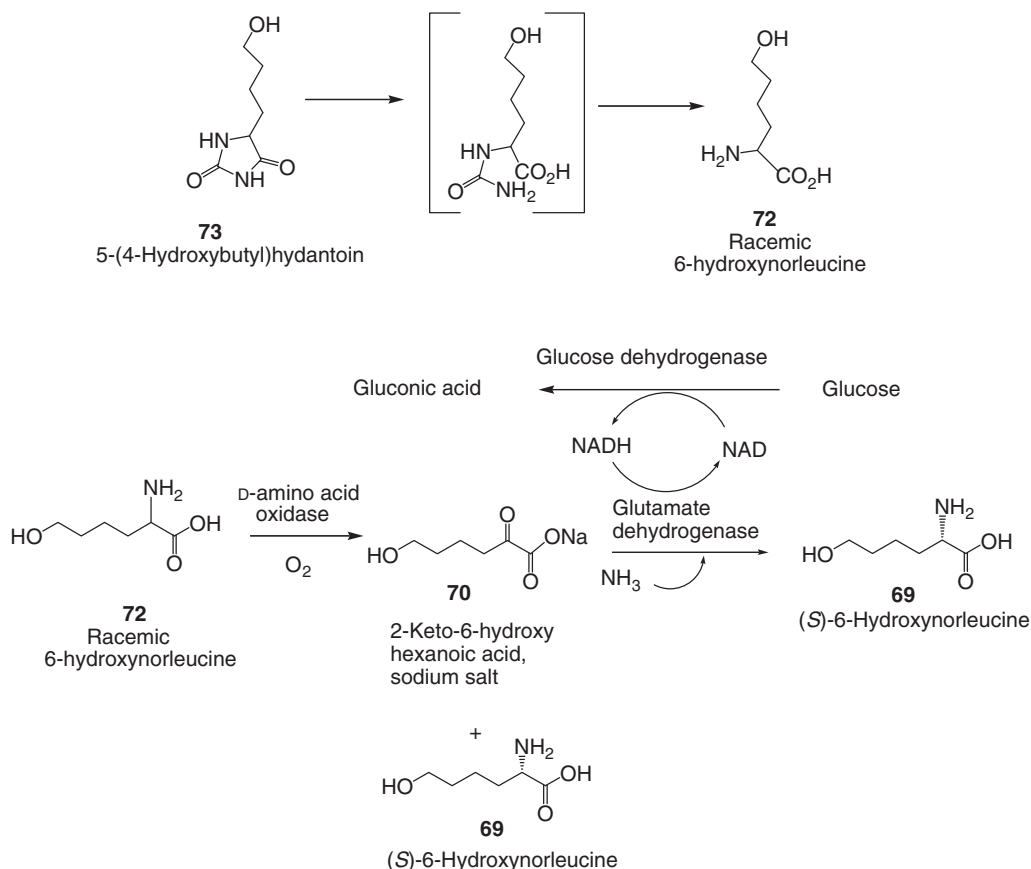


FIGURE 4.21 Conversion of racemic 6-hydroxynorleucine (**72**) to (*S*)-6-hydroxynorleucine (**69**) by (*R*)-amino acid oxidase and glutamate dehydrogenase.

increased to >99%, the reductive amination procedure was used to convert the mixture containing the 2-keto-6-(1,3-dioxolan-2-yl)hexanoic acid entirely to (*S*)-6-hydroxynorleucine in 97% yield with 98% ee from racemic 6-hydroxynorleucine at 100 g/L. Porcine kidney D-amino acid oxidase and beef liver catalase or *T. variabilis* whole cells (source of both the oxidase and catalase) were used successfully for this transformation [71]. The (*S*)-6-hydroxynorleucine prepared by the enzymatic process was converted chemically to omapatrilat (**68**) as described previously [72].

4.8.3.2 Enzymatic Synthesis of Allysine Ethylene Acetal

(*S*)-2-Amino-5-(1,3-dioxolan-2-yl)pentanoic acid [(*S*)-allysine ethylene acetal] (**74**, Figure 4.22) is one of the three building blocks used in an alternative synthesis of omapatrilat (**68**). It had been previously prepared following an eight-step chemical synthesis from 3,4-dihydro[2H]pyran [73]. An alternate synthesis of **74** was demonstrated by reductive amination of ketoacid acetal **75** using PDH from *T. intermedius* [74]. The reaction required ammonia and NADH; NAD produced during the reaction was recycled to NADH by the oxidation of formate to CO₂ using FDH. *T. intermedius* PDH was cloned and expressed in *E. coli*, inducible by β-D-isopropylthio-galactoside (IPTG). Fermentation of *T. intermedius* yielded 184 units of PDH activity per liter of whole broth in 6 h. In contrast, *E. coli* BL21 (DE3) (pPDH155K) produced over 19,000 units per liter of whole broth in about 14 h. *C. boidinii* [75] or *P. pastoris* [76] grown on methanol are useful sources of FDH. Expression of *T. intermedius* PDH in *P. pastoris*, inducible by methanol, allowed obtaining both enzymes from a single fermentation.

A procedure using heat-dried cells of *E. coli* containing cloned PDH and heat-dried *C. boidinii* was scaled up. A total of 197 kg of **74** was produced in three 1,600 L batches using a 5% concentration of substrate **75** with an average yield of 91% and an ee of >98%. A second-generation procedure, using dried recombinant *P. pastoris* containing *T. intermedius* PDH inducible with methanol and endogenous FDH, induced when *P. pastoris* was grown in a medium containing methanol, allowed both enzymes to be produced during a single fermentation. The procedure with *P. pastoris* was also scaled up to produce 15.5 kg of **74** in a maximum yield of 97% and >98% ee in a 180 L batch using 10% ketoacid **75** concentration. The (*S*)-allysine ethylene acetal (**74**) produced by the enzymatic process was converted to omapatrilat (**68**) [72].

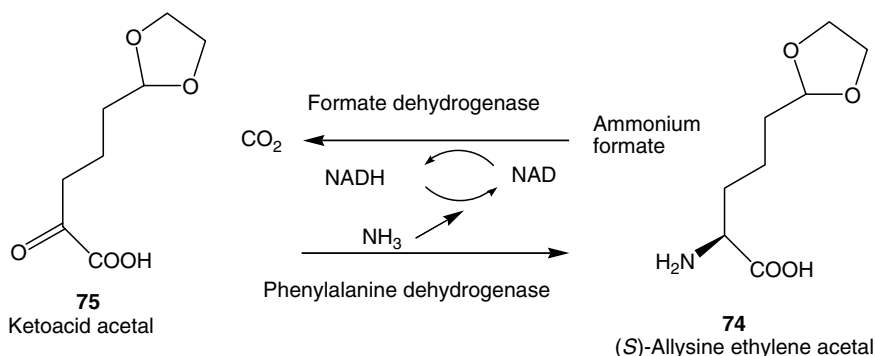


FIGURE 4.22 Enzymatic synthesis of chiral synthon for Omapatrilat (**68**): reductive amination of ketoacid acetal **75** to amino acid acetal **74** by phenylalanine dehydrogenase. Regeneration of NADH was carried out using formate dehydrogenase (FDH).

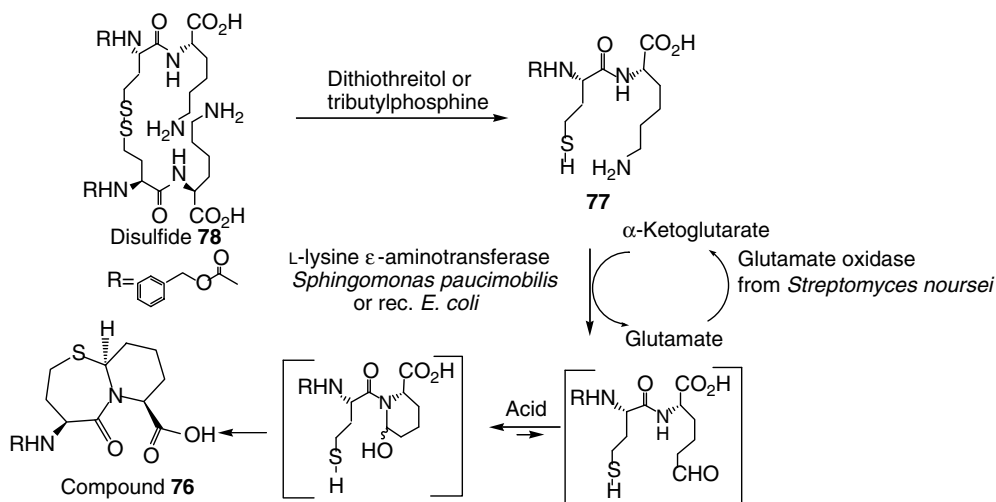


FIGURE 4.23 Enzymatic synthesis of chiral synthon for omapatrilat (**68**): conversion of disulfide **78** to thiazepine **76** by L-lysine ε-aminotransferase.

4.8.3.3 Enzymatic Synthesis of Thiazepine

[4*S*-(4*a*,7*a*,10*ab*)]-1-Octahydro-5-oxo-4-[[[(phenylmethoxy)carbonyl]amino]-7*H*-pyrido-[2,1-*b*] [1,3]thiazepine-7-carboxylic acid (**76**, Figure 4.23) is a key intermediate in the synthesis of omapatrilat (**68**) [67]. An enzymatic process was developed for the preparation of compound **76**. A selective culture technique was used to isolate eight different types of microbial cultures able to utilize *N*-α-Cbz-*S*-lysine as the sole source of nitrogen. Cell extracts were evaluated for oxidation of the ε-amino group of (*S*)-lysine in the thiol substrate **77** generated *in situ* from the disulfide *N*²-[*N*[(phenylmethoxy) carbonyl]-L-homocysteinyl]-L-lysine]-1,1-disulfide (**78**) by treatment with dithiothreitol (DTT). Product **76** formation was observed with four cultures. One of the cultures, Z-2, later identified as *Sphingomonas paucimobilis* SC 16113 was used for process development. Due to the low activity of enzyme [L-lysine ε-aminotransferase (LAT)] in *S. paucimobilis* SC 16113, and to minimize **77** hydrolysis, LAT was overexpressed in *E. coli* strain GI724(pAL781-LAT) and a biotransformation process was developed [77]. The aminotransferase reaction required α-ketoglutarate as the amine acceptor. Glutamate formed during this reaction was recycled back to α-ketoglutarate by glutamate oxidase (GOX) from *S. noursei* SC 6007. The extracellular GOX was cloned and expressed in *S. lividans*.

Biotransformation of compound **78** to compound **76** was carried out using LAT from *E. coli* GI724[pal781-LAT] in the presence of α-ketoglutarate and DTT (or tributylphosphine) and GOX. Maximum reaction yields of 65 to 67% were obtained. The reaction yield in the absence of GOX averaged only about 33 to 35%. However, the reaction yield increased to a maximum of 70%, by increasing the α-ketoglutarate to 40 mg/ml (10 × increase in concentration) and conducting the reaction at 40°C [77].

4.9 ANTICHOLESTROL DRUGS

4.9.1 MICROBIAL PRODUCTION OF (*S*)-4-CHLORO-3-OXOBUTANOATE ESTERS

Chiral β-hydroxy esters are versatile synthons in organic synthesis, specifically in the preparation of natural products [78,79]. The reduction of 4-chloro-3-oxobutanoic acid methyl ester (**79**) to (*S*)-4-chloro-3-hydroxybutanoic acid methyl ester (**80**, Figure 4.24) by cell

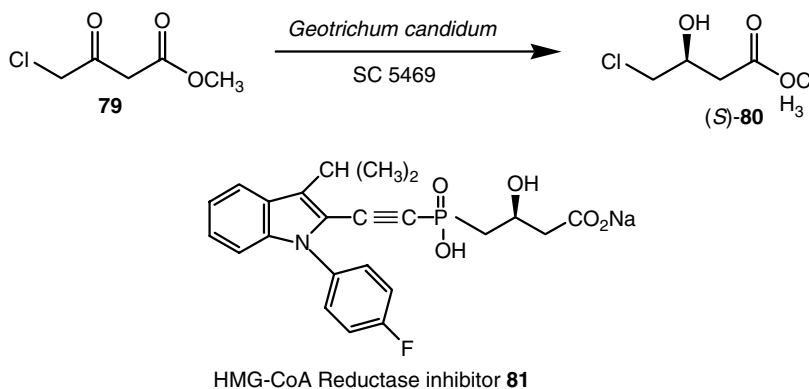


FIGURE 4.24 Synthesis of a chiral synthon for the cholesterol-lowering drug **81**: enantioselective microbial reduction of 4-chloro-3-oxobutanoic acid methyl ester **79** to (*S*)-4-chloro-3-hydroxybutanoic acid ester **80**.

suspensions of *Geotrichum candidum* SC 5469. *S*-(–)-**80** is a key chiral intermediate in the total chemical synthesis of **81**, a cholesterol antagonist that acts by inhibiting 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase [80]. In the biotransformation process, a reaction yield of 95% and ee of 96% were obtained for *S*-(–)-**80** by glucose-, acetate- or glycerol-grown cells (10% w/v) of *G. candidum* SC 5469 at 10 g/L substrate input. The ee of *S*-(–)-**80** was increased to 98% by heat treatment of cell suspensions (55°C for 30 min) prior to conducting the bioreduction of **79**.

4.9.2 ENZYMATIC PREPARATION OF (*S*)-4-CHLORO-3-HYDROXYBUTANOATE

In an alternate approach, the asymmetric reduction of ethyl 4-chloroacetoacetate to (*S*)-4-chloro-3-hydroxybutanoate was demonstrated by a secondary alcohol dehydrogenase (PfODH) from *P. finlandica*. The gene encoding PfODH was cloned from *P. finlandica* and overexpressed in *E. coli*. FDH was used to regenerate the cofactor NADH required for this reaction. With recombinant *E. coli* coexpressing both PfODH and FDH from *Mycobacterium* sp., (*S*)-4-chloro-3-hydroxybutanoate was produced in 98.5% yield and 99% ee at 32 g/L substrate input [81].

4.9.3 Enzymatic Preparation of (*R*)-4-Cyano-3-Hydroxybutyrate

An enzymatic process was developed for the preparation of 4-halo-3-hydroxybutyric acid derivatives by ketoreductase-catalyzed conversion of 4-halo-3-ketobutyric acid derivatives. Thus, the genes encoding halohydrin dehalogenase from *Agrobacterium tumefaciens*, ketoreductase from *C. magnoliae*, glucose dehydrogenase from *B. subtilis*, and FDH from *C. boidinii* were separately cloned into *E. coli* BL21. Each enzyme was then produced by fermentation, isolated, and characterized. Ethyl (*R*)-4-cyano-3-hydroxybutyrate was prepared from ethyl 4-chloroacetoacetate by the following procedure. Ethyl 4-chloroacetoacetate was incubated at pH 7.0 with ketoreductase, glucose dehydrogenase, and NADP for 40 h to produce ethyl (*S*)-chloro-3-hydroxybutyrate. The ethyl (*S*)-chloro-3-hydroxybutyrate was extracted with ethyl acetate, dried, filtered, and concentrated to yield a 97% ester. The dried ethyl (*S*)-chloro-3-hydroxybutyrate was dissolved in phosphate buffer and mixed with halohydrin dehalogenase and sodium cyanide at pH 8.0. After 57 h, (*R*)-4-cyano-3-hydroxybutyrate was recovered, an intermediate used in many HMG CoA reductase inhibitor syntheses [82].

4.9.4 ENZYMATIC PREPARATION OF (*R*)- AND (*S*)-ETHYL-3-HYDROXYBUTYRATE

An efficient two-step enzymatic process for production of (*R*)- and (*S*)-ethyl-3-hydroxybutyrate (HEB) was developed and scaled up to a multi-kg scale. Both enantiomers were obtained at 99% chemical purity and over 96% ee, with an overall process yield of 73%. The first reaction involved acetylation of racemic HEB with vinyl acetate for the production of (*S*)-HEB. In the second reaction, (*R*)-enriched ethyl-3-acetoxybutyrate (AEB) was subjected to alcoholysis with ethanol to derive optically pure (*R*)-HEB. Immobilized *C. antarctica* lipase B (CALB) was employed in both stages, with high productivity and selectivity. The type of butyric acid ester influenced the enantioselectivity of the enzyme. Thus, extending the ester alkyl chain from ethyl to octyl resulted in a decrease in ee, whereas using bulky groups, such as benzyl or *t*-butyl, improved the enantioselectivity of the enzyme. The immobilized enzyme was packed in a column and the reactants were circulated through the enzyme bed until the targeted conversion was reached. The desired products were separated from the reaction mixture in each of the two stages by fractional distillation. The main features of the process are the exclusion of solvent (thus ensuring high process throughput), and the use of the same enzyme for both the acetylation and the alcoholysis steps to prepare kilogram quantities of (*S*)-HEB and (*R*)-HEB [83].

4.9.5 ENZYMATIC SYNTHESIS OF ETHYL (3*R*,5*S*)-DIHYDROXY-6-(BENZYLOXY) HEXANOATE

The diol ethyl (3*R*,5*S*)-dihydroxy-6-(benzyloxy) hexanoate (**83a**, Figure 4.25) is a key intermediate in the synthesis of [4-[4 α ,6 β (*E*)]-6-[4,4-bis[4-fluorophenyl]3-(1-methyl-1H-tetrazol-5-yl)-1,3-butadienyl]tetrahydro-4-hydroxy-2H-pyren-2-one (**84**), a potential new

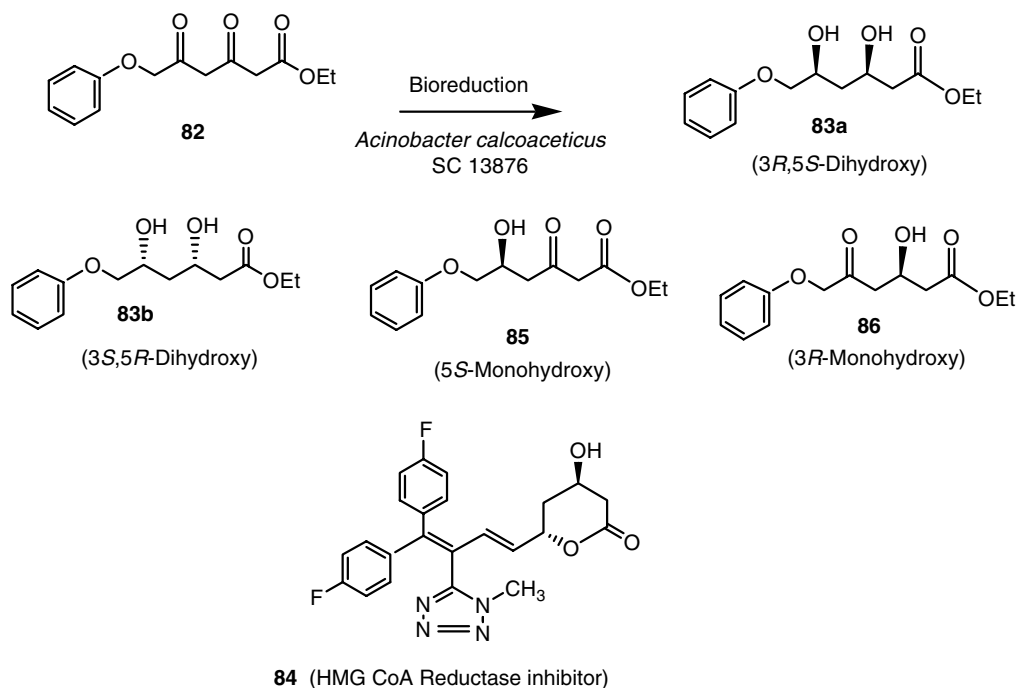


FIGURE 4.25 Synthesis of a chiral synthon for the cholesterol-lowering drug (5*R*,3*R*)-**84**: enantioselective microbial reduction of 3,5-dioxo-6-(benzyloxy)hexanoic acid, ethyl ester (**82**) to (3*S*,5*R*)-dihydroxy-6-(benzyloxy)hexanoic acid, ethyl ester (**83a**).

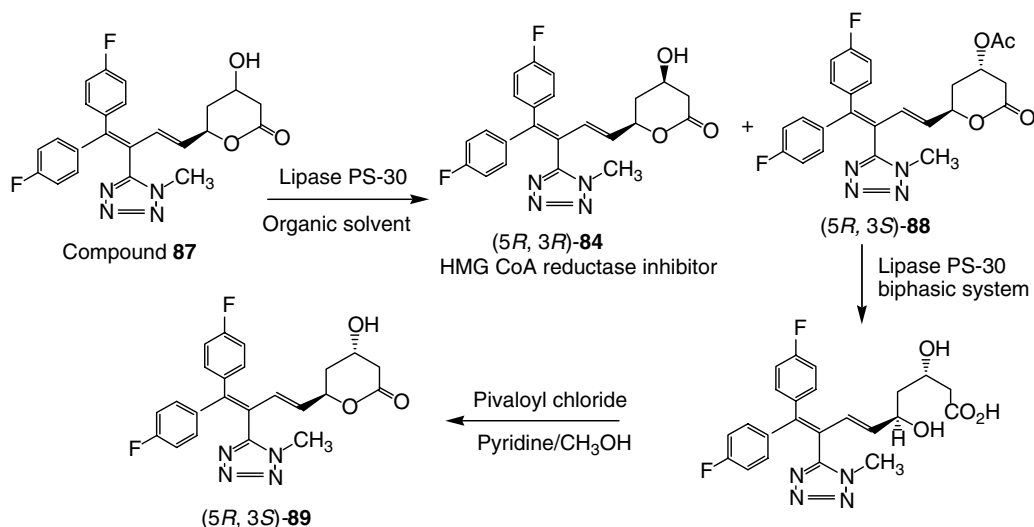


FIGURE 4.26 Synthesis of anticholesterol drug (5R,3R)-**84**: diastereoselective enzymatic acetylation of **87**.

anticholesterol drug that acts by inhibition of HMG CoA reductase [84]. The enantioselective reduction of the diketone ethyl 3,5-dioxo-6-(benzyloxy)hexanoate (**82**) to the diol ethyl (3R,5S)-dihydroxy-6-(benzyloxy)hexanoate (**83a**) [85] has been demonstrated by *Acinetobacter calcoaceticus* SC 13876 in a yield of 85% and diastereoselectivity of 97%.

Cell extracts of *A. calcoaceticus* SC 13876 in the presence of NAD⁺, glucose, and glucose dehydrogenase reduced **82** to the corresponding isomeric monohydroxy compounds **85** and **86**, which were further reduced to the compound **83a**. A reaction yield of 92% and a diastereomeric purity of 98% were obtained when the reaction was carried out at 10 g/L in a 1 L batch.

Using an enzymatic diastereoselective acetylation process, the (5R,3R)-alcohol **83a** (Figure 4.26) was prepared from **87** [86]. Lipase PS-30 and BMS lipase (produced by fermentation of *Pseudomonas* strain SC 13856) efficiently catalyzed the acetylation of the **87** (4 g/L) to yield the (5R,3S)-acetate **88** and unreacted desired (5R,3R)-alcohol **83a**. A maximum reaction yield of 49% and an ee of 98.5% were obtained for (5R,3R)-alcohol **83a** when the reaction was conducted in toluene in the presence of isopropenyl acetate as an acyl donor. In MEK at 50 g/L substrate concentration, a maximum reaction yield of 46% and an ee of 96% were obtained for **83a**. The enzymatic process was scaled up to a 640 L preparative batch using immobilized lipase PS-30. From the reaction mixture (5R,3R)-alcohol **83a** was isolated in 35% overall yield (theoretical maximum yield 50%) with 98.5% ee and 99.5% chemical purity. The (5R,3S)-acetate **88** produced by this process was enzymatically hydrolyzed by lipase PS-30 in a biphasic system to prepare the corresponding (5R,3S)-alcohol **89**.

4.9.6 ENZYMATIC PREPARATION OF A 2,4-DIDEOXYHEXOSE DERIVATIVE

The chiral 2,4-dideoxyhexose derivative required for the HMG CoA reductase inhibitors has also been prepared using 2-deoxyribose-5-phosphate aldolase (DERA). The reactions start with a stereospecific addition of acetaldehyde **90** (Figure 4.27) to a substituted acetaldehyde to form a 3-hydroxy-4-substituted butyraldehyde **91**, which reacts subsequently with another acetaldehyde to form a 2,4-dideoxyhexose derivative **92**. DERA has been expressed in *E. coli* [87].

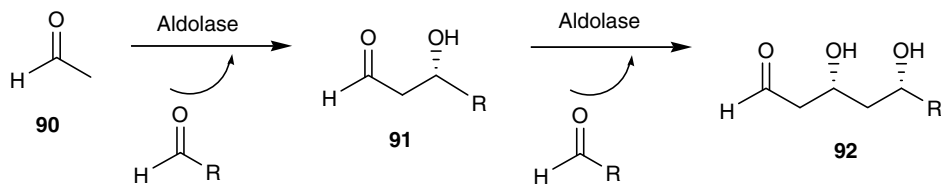


FIGURE 4.27 Enzymatic synthesis of 2,4-dideoxyhexose derivative **92**, a chiral synthon for anticholesterol drugs.

The above process has been improved and optimized. An improvement of almost 400-fold in volumetric productivity relative to the published enzymic reaction conditions has been achieved, resulting in an attractive process that has been run on up to a 100 g scale in a single batch at a rate of 30.6 g/L/h. The catalyst load has been improved tenfold as well, from 20 to 2.0 weight % DERA. These improvements were achieved by a combination of discovery of DERA with improved activity and reaction optimization to overcome substrate inhibition. The two stereogenic centers are set by DERA with ee at >99.9% and diastereomeric excess at 96.6%. In addition, downstream chemical processes have been developed to convert the enzymic product efficiently to versatile intermediates applicable to preparation of atorvastatin and rosuvastatin [88].

4.9.7 ENZYMATIC SYNTHESIS OF *S*-[1-(ACETOXYL)-4-(3-PHENYL)BUTYL]PHOSPHONIC ACID, DIETHYL ESTER

Squalene synthase is the first pathway-specific enzyme in the biosynthesis of cholesterol and catalyzes the head-to-head condensation of two molecules of farnesyl pyrophosphate (FPP) to form squalene. It has been implicated in the transformation of FPP into presqualene pyrophosphate (PPP). FPP analogs are a major class of inhibitors of squalene synthase [89]. However, this class of compounds lacks specificity and is a group of potential inhibitors of other FPP-consuming transferases such as geranyl geranyl pyrophosphate synthase. To increase enzyme specificity, analogs of PPP and other mechanism-based enzyme inhibitors, such as **94**, have been synthesized [90].

S-[1-(Acetoxy)-4-(3-phenyl) butyl]phosphonic acid, diethyl ester (**93**, Figure 4.28) is a key chiral intermediate required for the total chemical synthesis of **94**. The enantioselective acetylation of racemic [1-(hydroxy)-4-(3-phenyl)butyl]phosphonic acid, diethyl (**95**) has been demonstrated with *G. candidum* lipase in toluene using isopropenyl acetate as the acyl

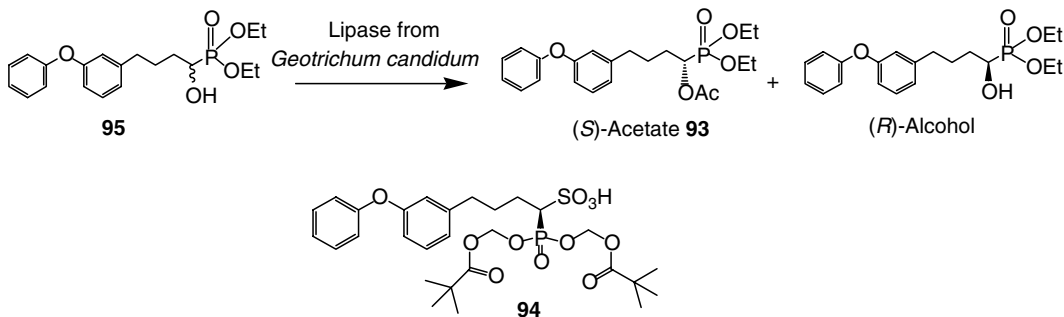


FIGURE 4.28 Enzymatic synthesis of a chiral synthon for the squalene synthase inhibitor **94**: enantioselective enzymatic acetylation of racemic **95** to (S)-acetate **93**.

donor [91]. A reaction yield of 38% (theoretical maximum yield 50%) and an ee of 95% were obtained for chiral **93**.

4.10 THROMBOXANE A2 ANTAGONIST

4.10.1 ENZYMATIC PREPARATION OF LACTOL [3*AS*-(3*α*,4*α*,7*α*,7*α*)]-4,7-EPOXYISOBENZOFURAN-1-(3*H*)-OL

Thromboxane A2 (TxA2) is an exceptionally potent vasoconstrictor substance produced by the metabolism of arachidonic acid in blood platelets and other tissues. Together with its potent antiaggregatory and vasodilator activities, TxA2 plays an important role in the maintenance of vascular homeostasis, and contributes to the pathogenesis of a variety of vascular disorders. Approaches towards limiting the effect of TxA2 have focused on either inhibiting its synthesis or blocking its action at its receptor sites by means of an antagonist [92,93]. The lactol [3*AS*-(3*α*,4*α*,7*α*,7*α*)]-hexahydro-4,7-epoxyisobenzofuran-1-(3*H*)-ol (**96**, Figure 4.29a) or the corresponding chiral lactone **97** are key intermediates in the total synthesis of [1*S*-[1*α*,2*α*(*Z*),3*α*,4*α* [[-7-[3-[[[1-oxoheptyl)-amine]acetyl]methyl]-7-oxabicyclo[2.2.1] hept-2-yl]-5-heptanoic acid (**98**), a new cardiovascular agent of potential use in the treatment of thrombotic disease [94].

The enantioselective oxidation of (exo,exo)-7-oxabicyclo[2.2.1]heptane-2,3-dimethanol (**99**) to the corresponding (*S*)-lactol **96** and (*S*)-lactone **97** has been demonstrated by *Nocardia globerula* ATCC 21505 and *Rhodococcus* sp. ATCC 15592 [95]. Lactone **97** was obtained in a maximum yield of 70% and 96% ee using cell suspensions of *N. globerula* ATCC 21505. An overall reaction yield of 46% (lactol and lactone combined) and ees of 96.7% and 98.4% were obtained for lactol **96** and lactone **97**, respectively, using cell suspensions of *Rhodococcus* sp. ATCC 15592.

The enantioselective hydrolysis of the diacetate (exo,exo)-7-oxabicyclo[2.2.1]heptane-2,3-dimethanol (**100**) to the corresponding *S*-monoacetate ester **101** (Figure 4.29b) has been demonstrated using lipase PS-30 from *P. cepacia* [96]. A maximum reaction yield of 75% and ee of >99% were obtained when the reaction was conducted in a biphasic system with 10% toluene. Lipase PS-30 was immobilized on Accurel polypropylene (PP) and the immobilized enzyme was reused (five cycles) without loss of enzymic activity, productivity, or ee of product **101**. The reaction process was scaled up to 80 L (400 g of substrate) and the product **101** was isolated in a maximum yield of 80% with 99.3% ee. The *S*-monoacetate was oxidized to its corresponding aldehyde, which was hydrolyzed to the (*S*)-lactol **96** used in the chemo-enzymatic synthesis of thromboxane A2 antagonist **98**.

4.11 CALCIUM CHANNEL BLOCKER

4.11.1 ENZYMATIC PREPARATION OF [(3*R*-*cis*)-1,3,4,5-Tetrahydro-3-Hydroxy-4-(4-Methoxyphenyl)-6-(Trifluoromethyl)-2*H*-1-Benzazepin-2-one]

Diltiazem **102** (Figure 4.30), a benzothiazepinone calcium channel-blocking agent that inhibits influx of extracellular calcium through L-type voltage-operated calcium channels, has been widely used clinically in the treatment of hypertension and angina [97]. Since diltiazem has a relatively short duration of action [98], an 8-chloro derivative has recently been introduced into the clinic as a more potent analog [99]. Lack of extended duration of action and little information on structure–activity relationships in this class of compounds led Floyd et al. [100] to prepare isosteric 1-benzazepin-2-ones; this led to the identification of (*cis*)-3-(acetoxy)-1-[2-(dimethylamino)ethyl]-1,3,4,5-tetrahydro-4-(4-methoxyphenyl)-6-trifluoromethyl)-2*H*-1-benzazepin-2-one (**103**) as a

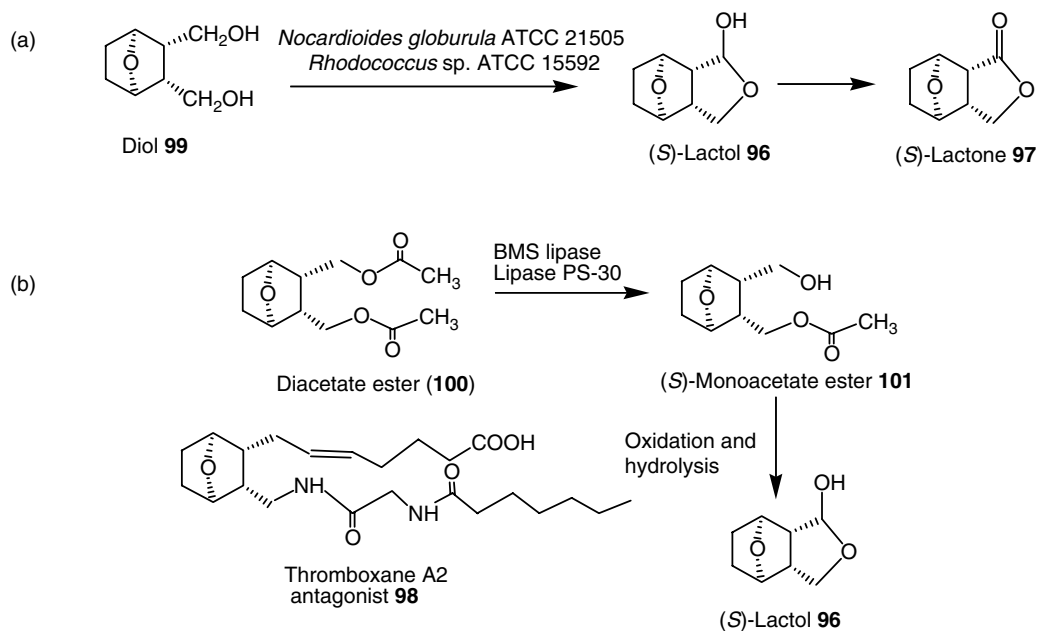


FIGURE 4.29 Synthesis of chiral synthon for thromboxane A2 antagonist **98**. (a) Stereoselective microbial oxidation of (exo,exo)-7-oxabicyclo[2.2.1]heptane-2,3-dimethanol (**99**) to the corresponding lactol **96** and lactone **97**. (b) Asymmetric enzymatic hydrolysis of (exo,exo)-7-oxabicyclo[2.2.1]heptane-2,3-dimethanol, diacetate ester (**100**) to the corresponding (S)-monoacetate ester **101**.

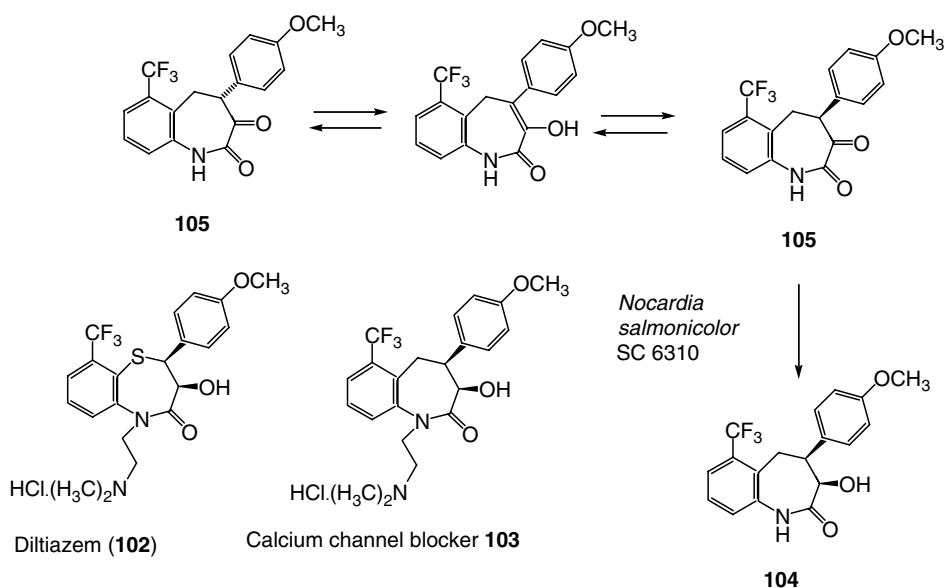


FIGURE 4.30 Synthesis of chiral synthon for calcium channel blocker **103**: microbial reduction of 4,5-dihydro-4-(4-methoxyphenyl)-6-(trifluoromethyl)-1H-benzazepin-2,3-dione (**105**).

longer-lasting and more potent antihypertensive agent. A key intermediate in the synthesis of this compound was (3*R*-*cis*)-1,3,4,5-tetrahydro-3-hydroxy-4-(4-methoxyphenyl)-6-(trifluoromethyl)-2*H*-1-benzazepin-2-one (**104**). An enantioselective process was developed for the reduction of 4,5-dihydro-4-(4-methoxyphenyl)-6-(trifluoromethyl)-1*H*-1-benzazepin-2,3-dione (**105**) to **104** using *N. salmonicolor* SC 6310, in 96% reaction yield with 99.8% ee [101].

4.12 POTASSIUM CHANNEL OPENER

4.12.1 MICROBIAL OXYGENATION TO PREPARE CHIRAL EPOXIDE AND DIOL

It has long been known that K channels play a major role in neuronal excitability and a critical role in the basic electrical and mechanical function of a wide variety of tissues, including smooth and cardiac muscle [102,103]. A new class of highly specific compounds that either open or block K channels has been developed [104]. The synthesis and antihypertensive activity of K-channel openers based on monosubstituted *trans*-4-amino-3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-3-ol (**106**, Figure 4.31) have been demonstrated [105,106]. Chiral epoxide **107** and diol **108** are potential intermediates for the synthesis of **106**. The enantioselective microbial oxygenation of 6-cyano-2,2-dimethyl-2*H*-1-benzopyran (**109**) to the corresponding chiral epoxide **107** and chiral diol **108** has been demonstrated [107]. *M. ramanniana* SC 13840 and *Corynebacterium* sp. SC 13876 gave maximum yields of 67.5% and 32% and ees of 96% and 89%, respectively, for (+)-*trans* diol **108**. *Corynebacterium* sp. SC 13876 also gave chiral epoxide **107** in a maximum yield of 17% and 88% ee.

A single-stage process (fermentation/epoxidation) for the biotransformation of **109** was developed using *M. ramanniana* SC 13840. In a 25 L fermentor, (+)-*trans* diol **108** was obtained in a maximum yield of 61% and ee of 92.5%. In a two-stage process using a cell suspension (10% w/v, wet cells) of *M. ramanniana* SC 13840, the (+)-*trans* diol **108** was obtained in a maximum yield of 76% with an ee of 96% when the reaction was carried out in a 5 L Bioflo fermentor. Glucose was supplied to regenerate NADH required for this reaction. From the reaction mixture, (+)-*trans* diol **108** was isolated in 65% overall yield with 97% ee and 98% chemical purity.

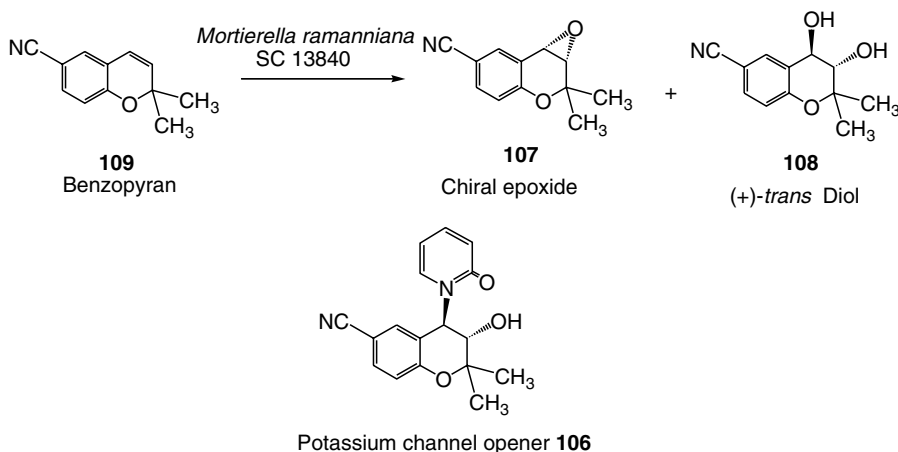


FIGURE 4.31 Preparation of chiral synthons for potassium channel openers **106**: oxygenation of 2,2-dimethyl-2*H*-1-benzopyran-6-carbonitrile (**109**) to the corresponding chiral epoxide **107** and (+)-*trans* diol **108** by *Mortierella ramanniana* SC 13840.

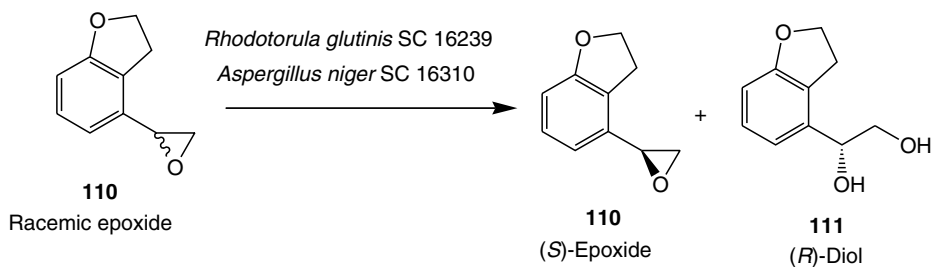


FIGURE 4.32 Synthesis of chiral intermediates for a melatonin receptor agonist: enantioselective microbial hydrolysis of racemic epoxide **110** to the corresponding (*R*)-diol **111** and unreacted (*S*)-epoxide **110**.

In an enzymatic resolution approach, (+)-*trans* diol **108** was prepared by the enantioselective acetylation of racemic diol with lipases from *C. cylindraceae* and *P. cepacia*. Both enzymes catalyzed the acetylation of the undesired enantiomer of the racemic diol to yield the monoacetylated product and unreacted (+)-*trans* diol **108**. A reaction yield of 40% (theoretical maximum yield 50%) and an ee of >90% were obtained with each lipase [108].

4.13 MELATONIN RECEPTOR AGONIST

4.13.1 ENANTIOSELECTIVE ENZYMATIC HYDROLYSIS OF RACEMIC 1-{2',3'-DIHYDRO BENZO[B]FURAN-4'-YL}-1,2-OXIRANE

Epoxide hydrolase catalyzes the enantioselective hydrolysis of an epoxide to the corresponding enantiomerically enriched diol and unreacted epoxide [109,110]. The (*S*)-epoxide **110** (Figure 4.32) is a key intermediate in the synthesis of a number of prospective drug candidates [111]. The enantiospecific hydrolysis of the racemic 1-{2', 3'-dihydro benzo[b]furan-4'-yl}-1,2-oxirane (**110**) to the corresponding (*R*)-diol **111** and unreacted *S*-epoxide **110** has been demonstrated [112]. Two *A. niger* strains (SC 16310 and SC 16311) and *Rhodotorula glutinis* SC 16293 selectively hydrolyzed the (*R*)-epoxide, leaving behind the (*S*)-epoxide **110** in >95% ee and 45% yield (theoretical maximum yield 50%). Several solvents at 10% v/v were evaluated in an attempt to improve the ee and yield. Solvents had significant effects on both the extent of hydrolysis and the ee of unreacted (*S*)-epoxide **110**. Most solvents gave a lower ee product and slower reaction rate than that of reactions without any solvent supplement, although MTBE gave a reaction yield of 45% (theoretical maximum yield 50%) and an ee of 99.9% for unreacted (*S*)-epoxide **110**.

4.13.2 BIOCATALYTIC DYNAMIC KINETIC RESOLUTION OF (*R,S*)-1-{2',3'-DIHYDROBENZO[B]FURAN-4'-YL}-ETHANE-1,2-DIOL

Most commonly used biocatalytic kinetic resolution of racemates often provide compounds with high ee, but the maximum theoretical yield of product is only 50%. The reaction mixture contains approximately 50:50 mixture of reactant and product that possesses only slight differences in physical properties (e.g., a hydrophobic alcohol and its acetate), and thus separation may be very difficult. These issues with kinetic resolutions can be addressed by employing a “dynamic kinetic resolution” process involving a biocatalyst or biocatalyst with metal-catalyzed *in situ* racemization [113,114].

S-1-{2',3'-Dihydrobenzo[b]furan-4'-yl}ethane-1,2-diol (**111**, Figure 4.33) is a potential precursor of *S*-epoxide **110** [112]. The dynamic kinetic resolution of the racemic diol **111** to

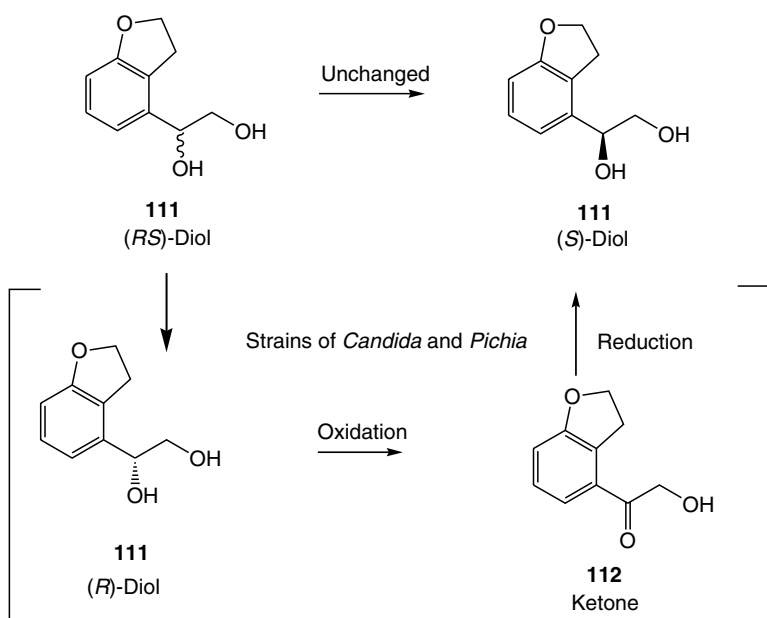


FIGURE 4.33 Synthesis of chiral intermediates for a melatonin receptor agonist: stereoinversion of racemic diol **111** to (*S*)-diol **111** by *Candida boidinii* and *Pichia methanolica*.

the (*S*)-enantiomer **111** has been demonstrated [115]. Seven cultures [*C. boidinii* SC 13821, SC 13822, SC 16115, *P. methanolica* SC 13825, SC 13860, and *H. polymorpha* SC 13895, SC 13896] were found to be promising, providing (*S*)-diol **111** in 87 to 100% ees and 60 to 75% yields. A new compound was formed during these biotransformations and was identified as the hydroxy ketone **112** from an LC-MS. The area of the high-performance liquid chromatography (HPLC) peak for hydroxy ketone **112** first increased with time, reached a maximum, and then decreased, as expected for the proposed dynamic kinetic resolution pathway. *C. boidinii* SC 13822, *C. boidinii* SC 16115, and *P. methanolica* SC 13860 transformed the racemic diol **111** in 3 to 4 days to (*S*)-diol **111** in >70% yield and 90 to 100% ee.

4.14 β -3-RECEPTOR AGONIST

β -3-Adrenergic receptors are found on the cell surfaces of both white and brown adipocytes and are responsible for lipolysis, thermogenesis, and relaxation of intestinal smooth muscle [116]. Consequently, several research groups are engaged in developing selective β -3 agonists for the treatment of gastrointestinal disorders, type II diabetes, and obesity [117,118]. Three different biocatalytic syntheses of chiral intermediates required for the total synthesis of β -3-receptor agonists **113** (Figure 4.34) have been demonstrated [119].

4.14.1 MICROBIAL REDUCTION OF 4-BENZYLOXY-3-METHANESULFONYLAMINO-2'-BROMOACETOPHENONE

The microbial reduction of 4-benzyloxy-3-methanesulfonylamino-2'-bromoacetophenone (**114**, Figure 4.34) to the corresponding (*R*)-alcohol **115** has been demonstrated [119] using *S. paucimobilis* SC 16113. The growth of *S. paucimobilis* SC 16113 was carried out in a 750 L fermentor and cells (60 kg) harvested from the fermentor were used to conduct the biotransformation in 10 L and 200 L preparative batches using 20% (w/v, wet cells).

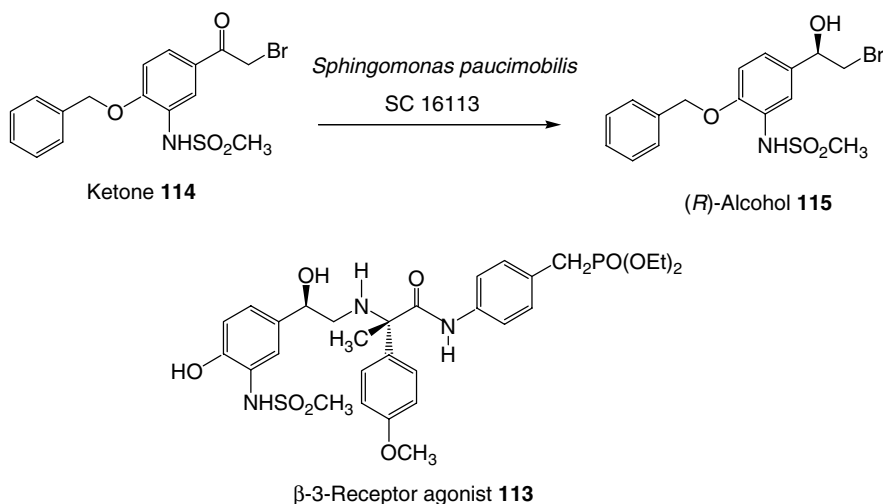


FIGURE 4.34 Enzymatic synthesis of chiral synthon for β -3-receptor agonist **113**: enantioselective reduction of 4-benzyloxy-3-methanesulfonylamino-2'-bromo-acetophenone (**114**) to (R)-alcohol **115**.

In some batches, the fermentation broth was concentrated threefold by microfiltration and subsequently washed with buffer by diafiltration and used directly in the bioreduction process. In all the batches, reaction yields of >85% and ees of >98% were obtained. The isolation of alcohol **115** from the 200 L batch gave 320 g (80% yield) of product with an ee of 99.5%.

In an alternate process, frozen cells of *S. paucimobilis* SC 16113 were used with XAD-16 hydrophobic resin (50 g/L) adsorbed substrate at 10 g/L concentration. In this process, an average reaction yield of 85% and an ee of >99% were obtained for alcohol **115**. At the end of the biotransformation, the reaction mixture was filtered on a 100 mesh (150 μ) stainless-steel screen, and the resin retained by the screen was washed with water. The product was then desorbed from the resin with acetonitrile and crystallized in a 75% overall yield and 99.8% ee.

4.14.2 ENZYMATIC RESOLUTION OF RACEMIC α -METHYLPHENYLALANINE AMIDES

The chiral amino acids **116** and **117** (Figure 4.35) are intermediates for the synthesis of β -3-receptor agonists [117,118]. These may be obtained by the enzymatic resolution of racemic α -methylphenylalanine amide **118** and α -methyl-4-methoxyphenylalanine amide **119**, respectively, by an amidase from *Mycobacterium neoaurum* ATCC 25795 [119]. Wet cells (10% w/v) completed resolution of amide **118** in 75 min with a yield of 48% (theoretical maximum yield 50%) and an ee of 95% for the desired (S)-amino acid **116**. Alternatively, freeze-dried cells were suspended in 100 mM potassium phosphate buffer (pH 7.0) at 1% concentration to give complete resolution in 60 min with a yield of 49.5% (theoretical maximum yield 50%) and an ee of 99% for the (S)-amino acid **116**.

Freeze-dried cells of *M. neoaurum* ATCC 25795 and partially purified amidase (amidase activity in cell extracts purified fivefold by diethyl aminoethyl cellulose column chromatography) were used for the biotransformation of compound **119**. A reaction yield of 49% and an ee of 78% were obtained for the desired product **117** using freeze-dried cells. The resolution was completed in 50 h. Using partially purified amidase, a reaction yield of 49% and an ee of 94% were obtained after 70 h.

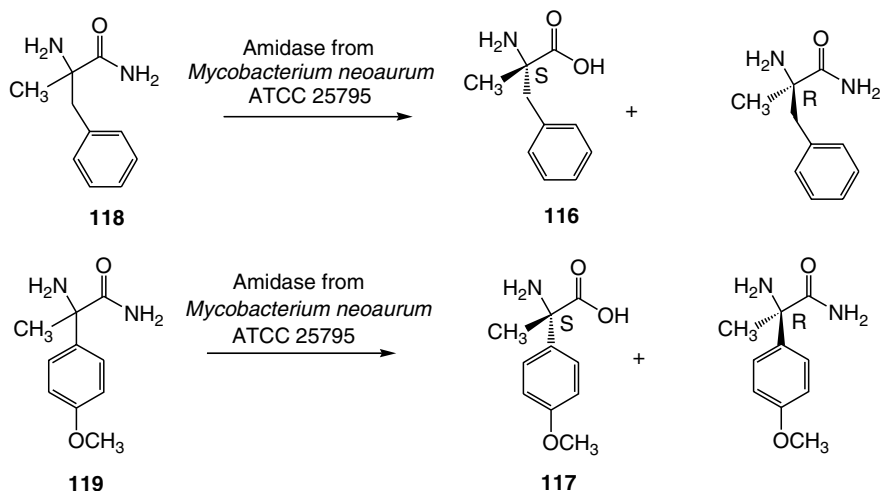


FIGURE 4.35 Enantioselective enzymatic hydrolysis of α -methyl phenylalanine amide (**118**) and α -methyl-4-hydroxyphenylalanine amide (**119**) by amidase.

4.14.3 ENANTIOSELECTIVE HYDROLYSIS OF DIETHYL METHYL-(4-METHOXYPHENYL)-PROPANEDIOATE

The (*S*)-monoester **120** (Figure 4.36) is a key intermediate for the synthesis of β -3-receptor agonists. The enantioselective enzymatic hydrolysis of diester **121** to the desired acid ester **120** by pig liver esterase [119] has been demonstrated. In various organic solvents the reaction yields and ees of monoester **120** were dependent upon the solvent used. High ees (>91%) were obtained with methanol, ethanol, and toluene as a cosolvent. Ethanol gave the highest reaction yield (96.7%) and ee (96%) for the desired acid ester **120**. It was observed that the ee of the (*S*)-monoester **120** was increased by decreasing the temperature from 25 to 10°C, when biotransformation was conducted in a biphasic system using ethanol as a cosolvent. A semipreparative 30 g scale hydrolysis was carried out using 10% ethanol as a cosolvent in a 3 L reaction mixture (pH 7.2) at 10°C for 11 h. A maximum reaction yield of 96% and an ee of 96.9% were obtained.

4.15 β -2-RECEPTOR AGONIST

4.15.1 ENANTIOSELECTIVE ENZYMIC ACYLATION

A potent β -2-receptor agonist formoterol **122** (Figure 4.37) is marketed as a diastereomeric mixture in spite of the varying efficacy of its stereoisomer. The preparation of the (*R,R*)-

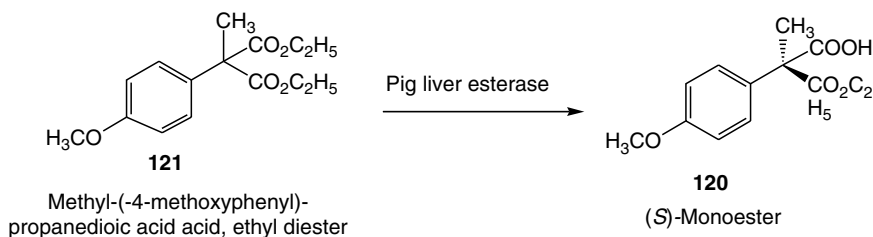


FIGURE 4.36 Enantioselective enzymatic hydrolysis of methyl-(4-methoxyphenyl)-propanedioic acid, ethyl diester (**121**) to (*S*)-monoester **120**.

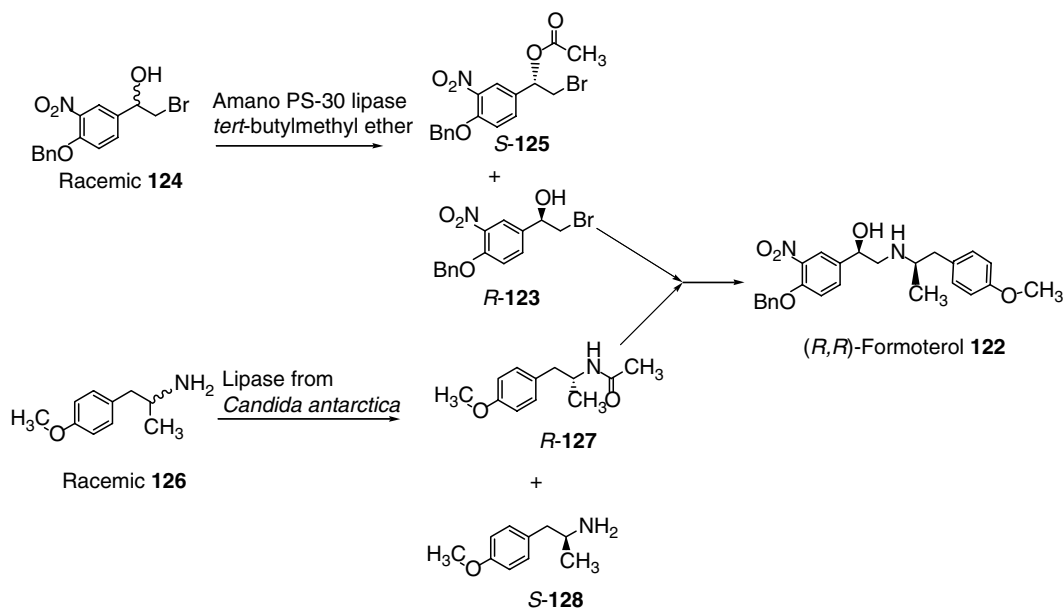


FIGURE 4.37 Enzymatic synthesis of chiral synthon for β -2-receptor-agonist **122**: enantioselective enzymatic acylation of racemic **124** and **126** to (*R*)-**123** and (*R*)-**127**.

stereoisomer was achieved by an enzymatic resolution process [120]. The *R*-bromohydrin **123** was prepared by enzymatic acylation of racemic alcohol **124** to yield acetylated product **S-125** and unreacted desired *R*-**123** (46% isolated yield) using lipase PS-30 from *P. cepacia*. The resolution of **126** was achieved by enzymatic acylation to yield desired *R*-**127** and unreacted *S*-**128** using *C. antarctic* lipase. An overall reaction yield of only 11% and an ee of 96% were obtained for *R*-**127**. Remarkably, the addition of 0.15 equivalent of triethylamine led to a 42% conversion to *R*-**127** in 94% ee in 4 h. Following hydrolysis and chromatography, *R*-**127** was isolated in 21% yield with 94% ee. Subsequently, coupling of the two chiral intermediates led to synthesis of (*R,R*)-formoterol.

4.16 TRYPTASE INHIBITOR

4.16.1 ENZYMATIC PREPARATION OF *S*-*N*-(*tert*-BUTOXYCARBONYL)-3-HYDROXYMETHYLPYPERIDINE

S-*N*-(*tert*-butoxycarbonyl)-3-hydroxymethylpiperidine (**129**, Figure 4.38) is a key intermediate in the synthesis of a potent tryptase inhibitor [121]. *S*-**129** was made from *R,S*-3-hydroxy methylpiperidine by fractional crystallization of the corresponding L(–)-dibenzoyl tartarate salt followed by hydrolysis and esterification [122]. Lipase from *P. cepacia* was found to be the best enzyme for the stereospecific resolution of *R,S*-*N*-(*tert*-butoxycarbonyl)-3-hydroxymethylpiperidine (**130**). *S*-**129** was obtained in 16% yield and >95% ee by hydrolysis of the *R,S*-acetate by lipase PS from *P. cepacia*. Lipase PS also catalyzed esterification of the *R,S*-*N*-(*tert*-butoxycarbonyl)-3-hydroxy methylpiperidine (**130**) with succinic anhydride (**131**) provided *R*-*N*-(*tert*-butoxycarbonyl)-3-hydroxy methylpiperidine (**132**) and the (*S*)-hemisuccinate ester **133**, which could be easily separated and hydrolyzed by base to the (*S*)-**129**. The

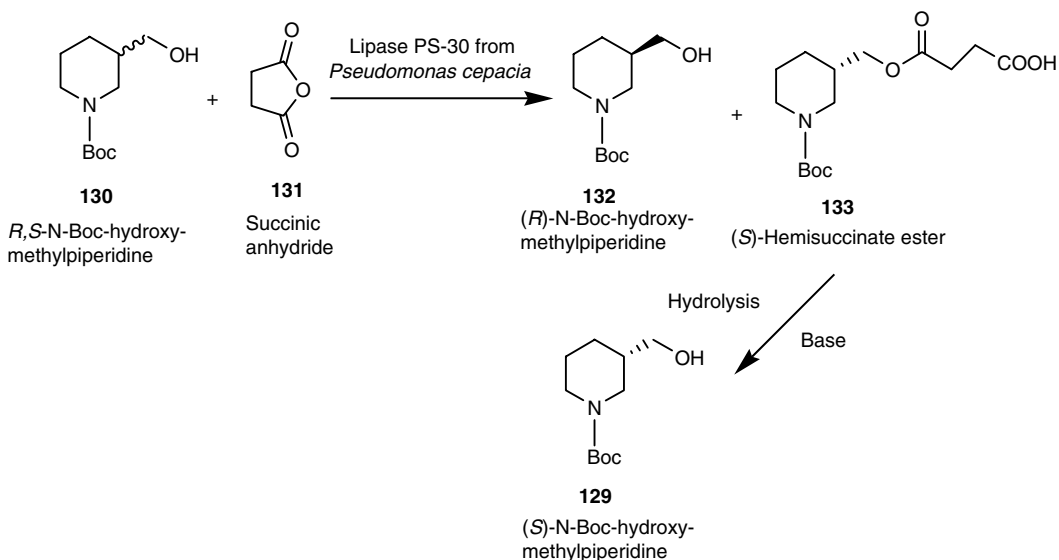


FIGURE 4.38 Preparation of a chiral synthon for a tryptase inhibitor: enzymatic resolution of racemic (*tert*-butoxycarbonyl)-3-hydroxymethylpiperidine (**130**) to (*S*)-**129**.

yield and ee could be improved greatly by repetition of the process. Using the repeated esterification/resolution procedure, (*S*)-**129** was obtained in 32% yield (maximum theoretical yield 50%) and 98.9% ee [123].

4.17 ANTI-ALZHEIMER'S DRUGS

4.17.1 ENZYMATIC PREPARATION OF (*S*)-2-PENTANOL AND (*S*)-2-HEPTANOL

(*S*)-2-Pentanol (Figure 4.39) is an intermediate in the synthesis of several potential anti-Alzheimer's drugs that inhibit β -amyloid peptide release and/or its synthesis [124,125]. The enzymatic resolution of racemic 2-pentanol and 2-heptanol by lipase B from *C. antarctica* has been demonstrated [126].

Commercially available lipases were screened for the enantioselective acetylation of racemic 2-pentanol in an organic solvent (hexane) in the presence of vinyl acetate as an acyl donor. *C. antarctica* lipase B gave a reaction yield of 49% (theoretical maximum yield 50%) and 99% ee for (*S*)-2-pentanol at 100 g/L substrate input. Among acylating agents tested, succinic anhydride was found to be of choice due to easy recovery of the (*S*)-2-pentanol at the end of the reaction. Reactions were carried out using racemic 2-pentanol as solvent as well as substrate. Using 0.68 mol equivalent of succinic anhydride and 13 g of lipase B per kg of racemic 2-pentanol, a maximum reaction yield of 43% and an ee of >98% were obtained for (*S*)-2-pentanol, isolated in 38% overall yield. The resolution of 2-heptanol was also carried out using lipase B under similar conditions to give a maximum reaction yield of 44% and an ee of >99% of (*S*)-2-heptanol, isolated in 40% overall yield.

In an alternate approach, the enantioselective reduction of 2-pentanone to the corresponding (*S*)-2-pentanol (Figure 4.39) has been demonstrated by *Gluconobacter oxydans*. Using triton X-100 treated cells of *G. oxydans*, preparative scale reduction of 2-pentanone was carried out and 1.06 kg of (*S*)-2-pentanol was prepared [127].

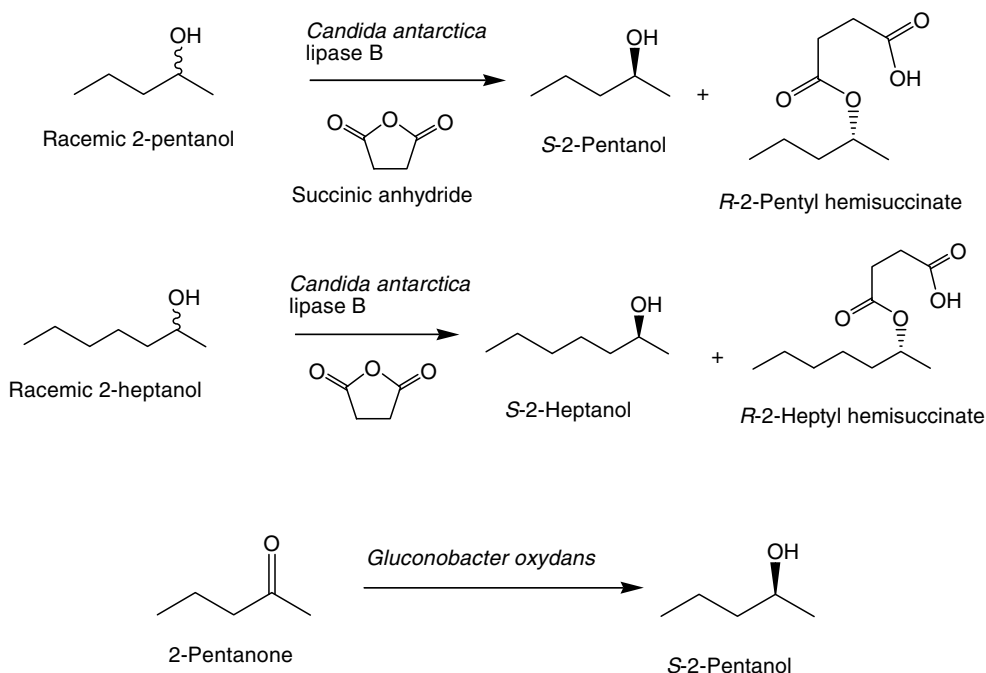


FIGURE 4.39 Synthesis of chiral intermediates for anti-Alzheimer's drugs: enzymatic resolution of racemic 2-pentanol and 2-heptanol by *Candida antarctica* lipase and enantioselective microbial reduction of 2-pentanone to (*S*)-2-pentanol.

4.17.2 ENANTIOSELECTIVE ENZYMATIC REDUCTION OF 5-OXOHEXANOATE AND 5-OXOHEXANENITRILE

Ethyl-(*S*)-5-hydroxyhexanoate (**134**) and (*S*)-5-hydroxyhexanenitrile (**135**, Figure 4.40) are key chiral intermediates in the synthesis of pharmaceuticals. Both chiral compounds have been prepared by enantioselective reduction of ethyl-5-oxohexanoate (**136**) and 5-oxohexanenitrile (**137**) by *P. methanolica* SC 16116. Reaction yields of 80 to 90% and >95% ees were obtained for each chiral compound. In an alternate approach, the enzymatic resolution of racemic 5-hydroxyhexanenitrile (**138**) by enzymatic succinylation was demonstrated using immobilized lipase PS-30 to obtain (*S*)-5-hydroxyhexanenitrile (**135**) in 35% yield (theoretical maximum yield 50%). (*S*)-5-Acetoxyhexanenitrile (**139**) was prepared by enantioselective enzymatic hydrolysis of racemic 5-acetoxyhexanenitrile (**140**) by *C. antarctica* lipase. A reaction yield of 42% and an ee of >99% were obtained [128].

4.17.3 ENANTIOSELECTIVE MICROBIAL REDUCTION OF SUBSTITUTED ACETOPHENONE

The chiral intermediate (*S*)-1-(2'-bromo-4'-fluoro phenyl)ethanol (**142**, Figure 4.41) was prepared by the enantioselective microbial reduction of 2-bromo-4-fluoro acetophenone (**141**) [129]. Organisms from genus *Candida*, *Hansenula*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Sphingomonas*, and Baker's yeast reduced **141** to **142** in >90% yield and 99% ee.

In an alternate approach, the enantioselective microbial reduction of methyl-, ethyl- and *tert*-butyl-4-(2'-acetyl-5'-fluorophenyl) butanoates **143**, **145**, and **147**, respectively, was demonstrated using strains of *Candida* and *Pichia*. Reaction yields of 40 to 53% and ees of 90 to 99% were obtained for the corresponding (*S*)-hydroxy esters **144**, **146**, and **148**. The reductase

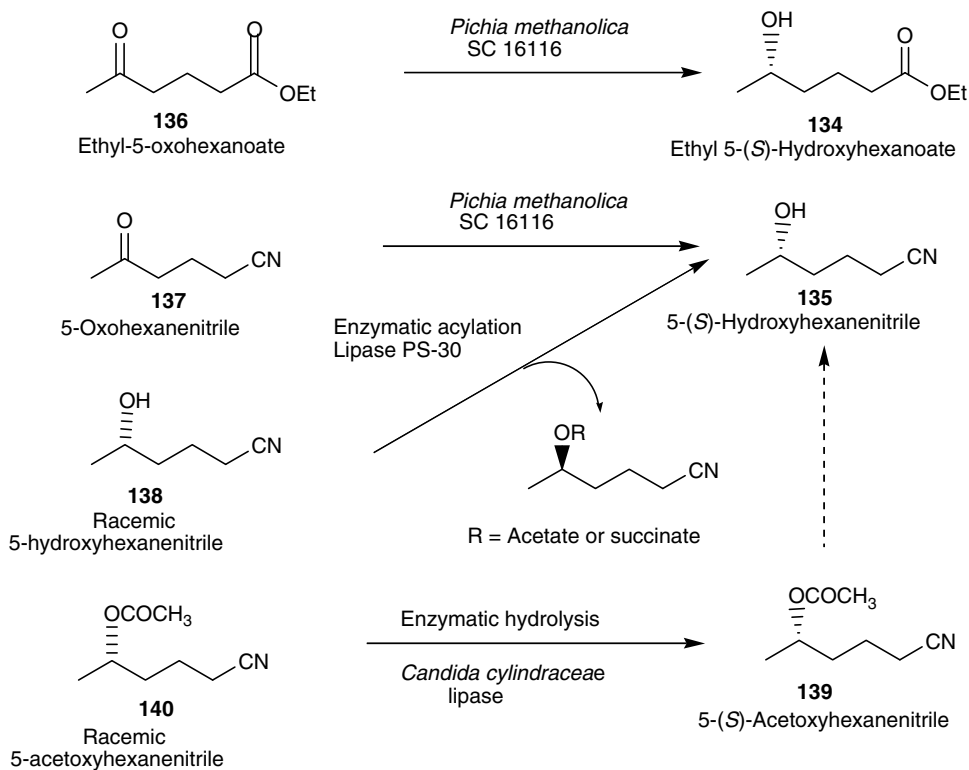


FIGURE 4.40 Synthesis of chiral intermediates for anti-Alzheimer's drugs: enantioselective microbial reduction of ethyl-5-oxohexanoate (**136**) and 5-oxohexanenitrile (**137**) and enzymatic resolution of 5-hydroxyhexanenitrile (**138**) and 5-acetoxyhexanenitrile (**140**).

that catalyzed the enantioselective reduction of ketoesters was purified to homogeneity from cell extracts of *P. methanolica* SC 13825. It was cloned and expressed in *E. coli* and recombinant cultures were used for the enantioselective reduction of the keto-methyl ester **143** to the corresponding (*S*)-hydroxy methyl ester **144**. On a preparative 300 L scale, a reaction yield of 98% with an ee of 99% was obtained [129].

4.18 RETINOID RECEPTOR GAMMA-SPECIFIC AGONISTS

4.18.1 ENZYMATIC PREPARATION OF 2-(*R*)-HYDROXY-2-(1',2',3',4'-TETRAHYDRO-1',1',4',4'-TETRAMETHYL-6'-NAPHTHALENYL) ACETATE

A number of studies have demonstrated that retinoids (vitamin A derivatives) are essential for normal growth, vision, tissue homeostasis, and reproduction [130]. Retinoic acid and its natural and synthetic analogs (retinoids) exert a wide variety of biological effects by binding to, or activating, a specific receptor or sets of receptors [131]. They have been shown to effect cellular growth and differentiation, and are promising drugs for the treatment of cancers [132]. A few retinoids are already in clinical use for the treatment of dermatological diseases such as acne and psoriasis [133]. (*R*)-3-Fluoro-4-[[hydroxy-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-acetyl]amino]benzoic acid **149** (Figure 4.42) is a retinoic acid receptor gamma-specific agonist potentially useful as a dermatological and anticancer drug [134].

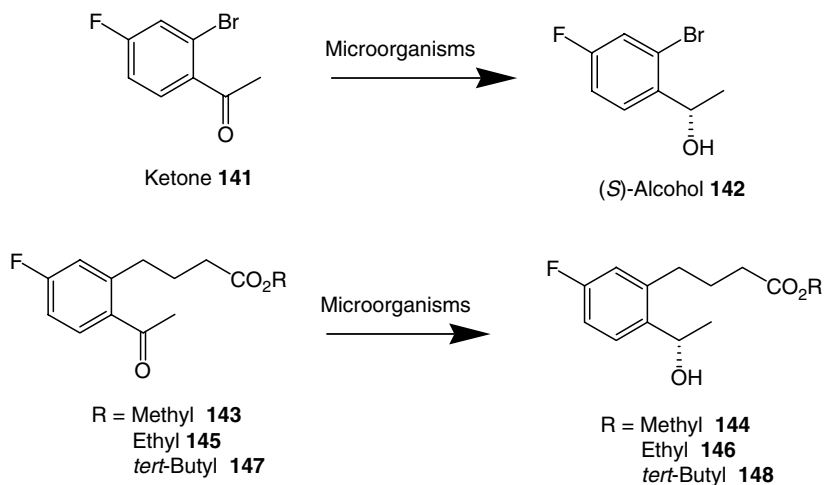


FIGURE 4.41 Synthesis of chiral intermediates for anti-Alzheimer's drugs: enantioselective microbial reduction of 2-bromo-4-fluoro acetophenone (**141**) and methyl 4-(2'-acetyl-5'-fluorophenyl) butanoate (**143**).

Ethyl 2-(*R*)-hydroxy-2-(1',2',3',4'-tetrahydro-1',1',4',4'-tetramethyl-6'-naphthalenyl)acetate (**150**) and the corresponding acid **151** were prepared as intermediates in the synthesis of the retinoic acid receptor gamma-specific agonist **149** [135]. Enantioselective microbial reduction of ethyl 2-oxo-2-(1',2',3',4'-tetrahydro-1',1',4',4'-tetramethyl-6-naphthalenyl) acetate

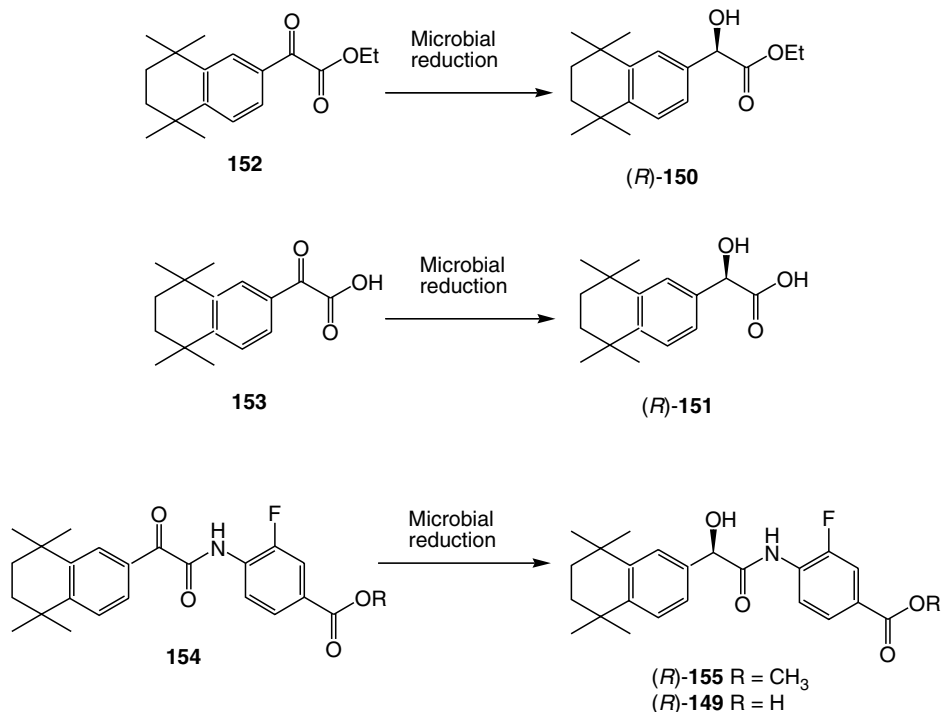


FIGURE 4.42 Enzymatic synthesis of chiral synthons for the retinoid receptor gamma-specific agonist **149**: enantioselective microbial reduction of ketoester **152**, ketoacid **153**, and ketoamide **154**.

(**152**) to alcohol **150** was carried out using *Aureobasidium pullulans* SC 13849 in 98% yield with an ee of 96%. At the end of the reaction, hydroxyester **150** was adsorbed onto XAD-16 resin and, after filtration, recovered in 94% yield from the resin with acetonitrile extraction. The recovered (*R*)-hydroxyester **150** was treated with Chirazyme L-2 or pig liver esterase to convert it to the corresponding (*R*)-hydroxyacid **151** in quantitative yield.

Among microorganisms screened for the reduction of 2-oxo-2-(1',2',3',4'-tetrahydro-1',1',4',4'-tetramethyl-6'naphthalenyl)acetic acid (**153**) to hydroxy acid **151**, *C. maltosa* SC 16112 and two strains of *C. utilis* (SC 13983, SC 13984) gave >53% reaction yields with >96% ee. The enantioselective microbial reduction of ketoamide **154** to the corresponding (*R*)-hydroxyamide **155** by *A. pullulans* SC 13849 was also demonstrated [135].

4.19 ANTI-INFECTIVE DRUGS

4.19.1 MICROBIAL HYDROXYLATION OF PLEUROMUTILIN OR MUTILIN

Pleuromutilin (**156**, Figure 4.43) is an antibiotic from *Pleurotus* or *Clitopilus* basidiomycetes strains that kills mainly gram-positive bacteria and mycoplasmas. A more active semisynthetic analog, tiamulin, has been developed for the treatment of animals and poultry infection and has been shown to bind to prokaryotic ribosomes and inhibit protein synthesis [136]. Metabolism of pleuromutilin derivatives results in hydroxylation by microsomal cytochrome P-450 at the 2- or 8-position and inactivates the antibiotics [137]. Modification of the 8-position of pleuromutilin and its analogs is of interest as a means of preventing the metabolic hydroxylation. Microbial hydroxylation of pleuromutilin **156** or mutilin **157** would provide a functional group at this position to allow further modification. The target analogs would maintain the biological activity of the parent compounds but not be susceptible to metabolic inactivation.

Biotransformation of mutilin and pleuromutilin by microbial cultures has been investigated to provide a source of 8-hydroxymutilin or 8-hydroxypleuromutilin [138]. *S. griseus* strains SC 1754 and SC 13971 (ATCC 13273) hydroxylated mutilin to 2-(*S*)-hydroxymutilin (**158**), 7-(*S*)-hydroxymutilin (**159**), and 8-(*S*)-hydroxymutilin (**160**, Figure 4.43). *Cunninghamella echinulata* SC 16162 (NRRL 3655) gave (2*S*)-hydroxymutilin and (2*R*)-hydroxypleuromutilin (**161**) from biotransformation of mutilin and pleuromutilin, respectively. The biotransformation of mutilin by the *S. griseus* strain SC 1754 was scaled up in 15, 60, and 100 L fermentations to produce a total of 49 g of (8*S*)-hydroxymutilin, 17 g of (7*S*)-hydroxymutilin, and 13 g of (2*S*)-hydroxymutilin from 162 g of mutilin [138].

A C-8 ketopleuromutilin **162** derivative has been synthesized from the biotransformation product 8-hydroxymutilin [139]. A key step in the process was the selective oxidation at C-8 of 8-hydroxymutilin using tetrapropylammonium perruthenate. The presence of the C-8 keto group precipitated interesting intramolecular chemistry to afford **163** with a novel pleuromutilin-derived ring system by acid catalyzed conversion of C-8 ketopleuromutilin.

4.19.2 ENZYMATIC PREPARATION OF (*R*)-1,3-BUTANEDIOL AND (*R*)-4-CHLORO-3-HYDROXYBUTONOATE

(*R*)-1,3-butanediol (**164**, Figure 4.44) is a key starting material of azetidinone derivatives **165**, which are key chiral intermediates for the syntheses of penem **166** and carbapenem antibiotics [140]. From a microbial screen the *C. parapsilosis* strain IFO 1396 was identified which produced (*R*)-1,3-butanediol from the racemate. The (*S*)-1,3-butanediol oxidizing enzyme (CpSADH), which produced (*R*)-1,3-butanediol from the racemate, was cloned in *E. coli*. The recombinant culture catalyzed the enantioselective oxidation of secondary alcohols and also

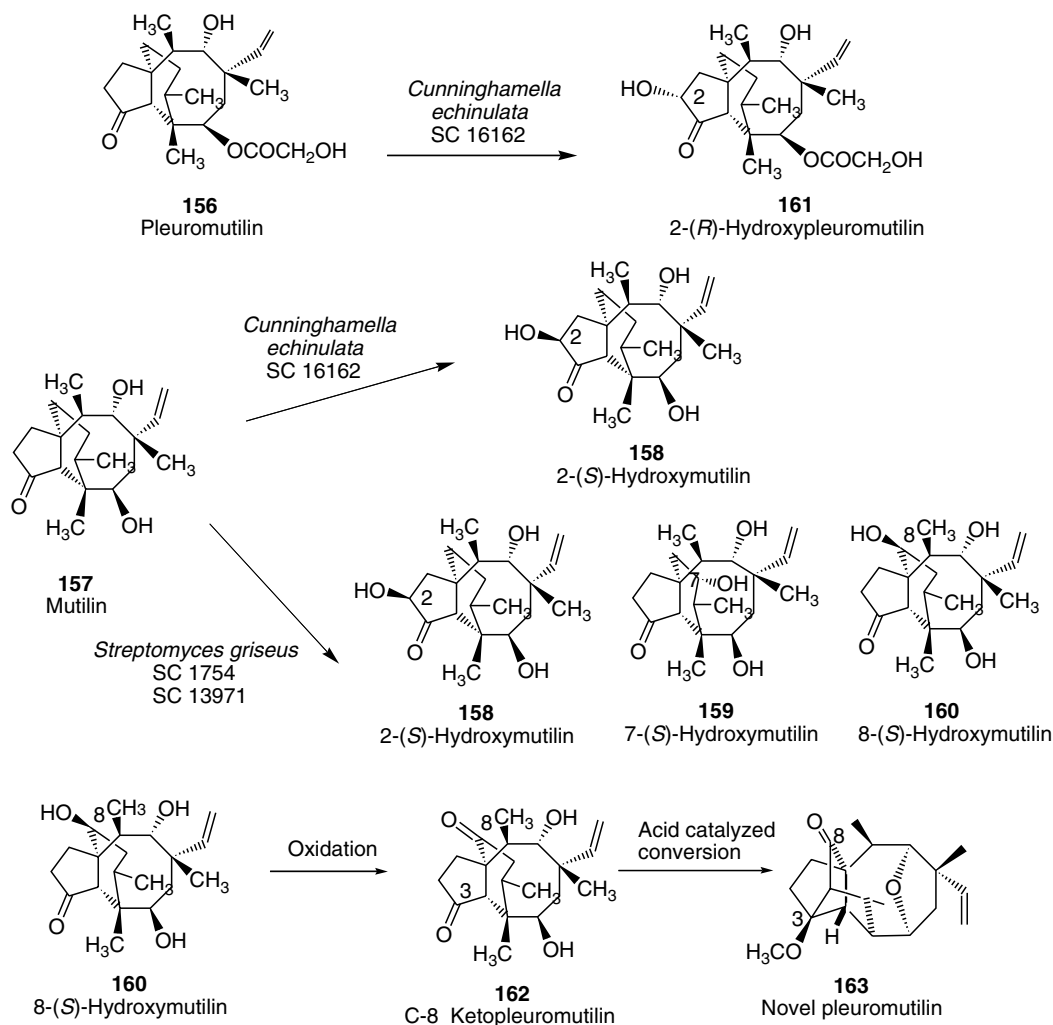


FIGURE 4.43 Microbial hydroxylation of pleuromutilin (**156**) and mutilin (**157**).

catalyzed the asymmetric reduction of aromatic and aliphatic ketones to their corresponding (*S*)-secondary alcohols. Using the recombinant enzyme, (*R*)-1,3-butanediol was produced in 97% yield and 95% ee using 150 g/L input of the racemate. Recombinant enzyme (CpSADH) was also used for reduction of ethyl 4-chloroacetoacetate (**167**) to produce (*R*)-4-chloro-3-hydroxybutanoate (**168**) in 95% yield and 99% ee using 36 g/L substrate input. Isopropanol was used to regenerate the NADH required for this reduction. (*R*)-4-Chloro-3-hydroxybutanoate is useful for the synthesis of L-carnitine (**169**) and (*R*)-4-hydroxypyrrolidone (**170**) [141].

4.19.3 ENZYMIC SYNTHESIS OF L- β -HYDROXYVALINE

The asymmetric synthesis of β -hydroxy- α -amino acids by various methods has been demonstrated [142–144] because of their utility as starting materials for the total synthesis of monobactam antibiotics. L- β -hydroxyvaline **171** is a key chiral intermediate required for the total synthesis of orally active monobactam [145], Tigemonam **172** (Figure 4.45). The

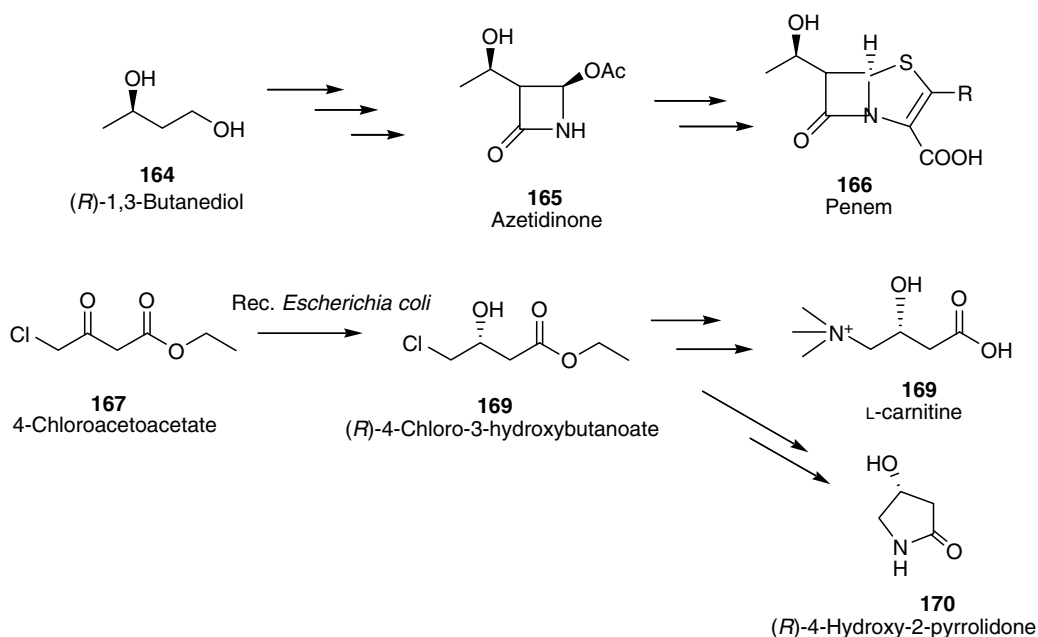


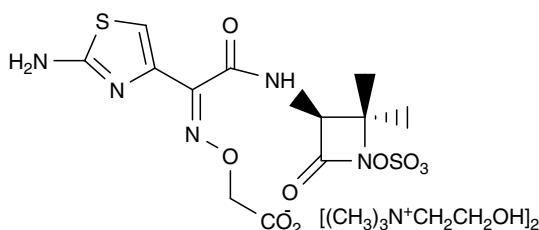
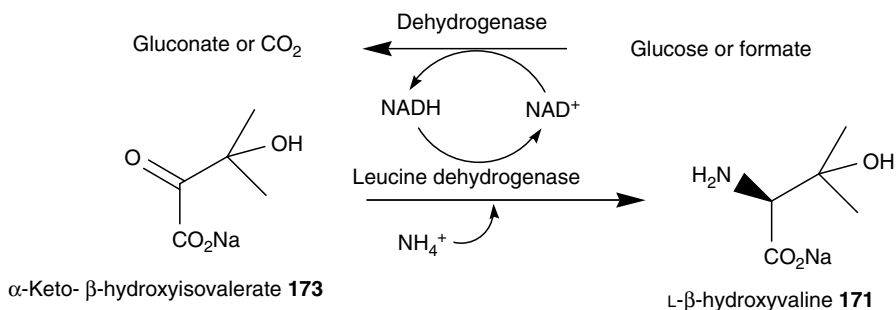
FIGURE 4.44 Enzymatic resolution of 1,3-butanediol and enantioselective enzymatic reduction of ethyl 4-chloroacetoacetate (**167**).

resolution of Cbz- β -hydroxyvaline by chemical methods has been demonstrated [146]. The synthesis of L- β -hydroxyvaline **171** from α -keto- β -hydroxyisovalerate **173** by reductive amination using leucine dehydrogenase from *B. sphaericus* ATCC 4525 has been demonstrated (Figure 4.45) [147]. NADH required for this reaction was regenerated by either FDH from *C. boidinii* or glucose dehydrogenase from *B. megaterium*. The immobilized cofactors such as polyethylene glycol-NADH and dextrans-NAD were effective in the biocatalytic process. The required substrate **173** was generated either from α -keto- β -bromoisovalerate or its ethyl esters by hydrolysis with sodium hydroxide *in situ*. In an alternate approach, the substrate **173** was also generated from methyl-2-chloro-3,3-dimethyloxiran carboxylate and the corresponding isopropyl and 1,1-dimethylethyl ester. These glycidic esters are converted to substrate **173** by treatment with sodium bicarbonate and sodium hydroxide. In this process, an overall reaction yield of 98% and an ee of 99.8% were obtained for the L- β -hydroxyvaline **171**.

4.20 α 1-ADRENORECEPTOR ANTAGONIST

4.20.1 ENZYMATIC ESTERIFICATION AND AMMONOLYSIS

Afuzosin (**174**, Figure 4.46), a quinoxaline derivative, acts as a potent and selective antagonist of α 1-adrenoreceptor-mediated contraction of the prostate and the prostatic capsule, thereby reducing the symptoms associated with benign prostatic hypertrophy [148]. Several routes have been reported for the chemical synthesis of Afuzosin, with tetrahydro-*N*-[3-(methylamino)-propyl]-2-furancarboxamide (**175**) as a widely used intermediate [149]. Its synthesis from 2-tetrahydrofuroic acid is difficult, involving toxic reagents and drastic reaction conditions [150]. Lipase-catalyzed ammonolysis reactions using ammonia as the nucleophile have been demonstrated with esters [151]. A lipase-catalyzed process has been described for the



Tigemonam **172**

FIGURE 4.45 Enzymatic preparation of L- β -hydroxyvaline **171**, an intermediate for synthesis of Tigemonam **172**.

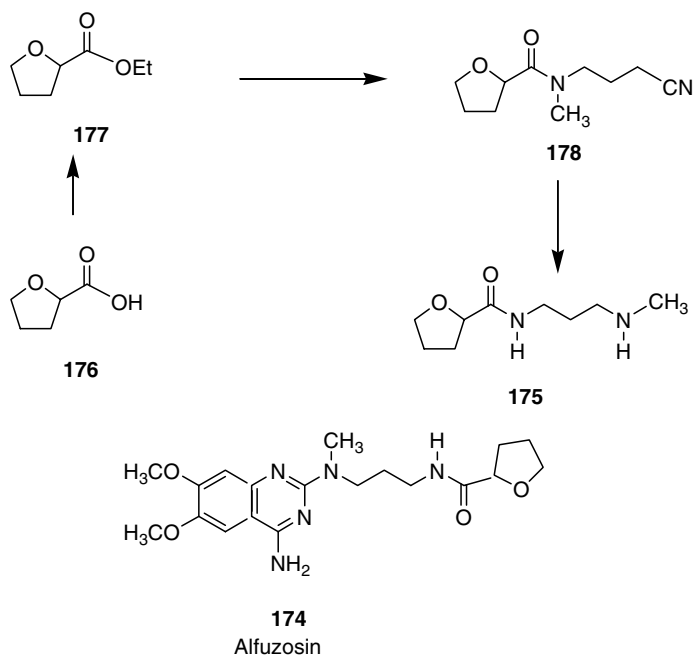


FIGURE 4.46 Preparation of a chiral synthon for an α 1-adrenoreceptor antagonist **174**: enzymatic synthesis of tetrahydro-*N*-[3-(methylamino)-propyl]-2-furancarboxamide (**175**) from carboxylic acid **176**.

one-pot conversion of carboxylic acids into substituted amides through *in situ* formation of the ethyl ester and subsequent ammonolysis [152]. The procedure was optimized for the preparation of **175** and involved the treatment of the corresponding carboxylic acid **176** with ethyl alcohol to prepare ester **177** in the presence of immobilized *C. antarctica* lipase followed by addition of *N*-methyl-1,3-propanediamine. The amide **175** was obtained in 72% yields. Immobilized enzyme was reused over eight cycles in this process.

This process was proven to be general and can be applied to open-chain, cyclic, hydroxy-, amino-, dicarboxylic, and unsaturated acids [152]. The enzyme shows regioselective behavior in relation to primary and secondary amino groups.

4.21 ENDOTHELIN RECEPTOR ANTAGONIST

4.21.1 ENANTIOSELECTIVE MICROBIAL REDUCTION OF KETO ESTER AND CHLOROKETONE

Endothelin is present in elevated levels in the blood of patients with hypertension, acute myocardial infraction, and pulmonary hypertension. Two endothelin receptor subtypes have been identified that bind endothelin, thus causing vasoconstriction [153,154]. Endothelin antagonists such as compound **179** (Figure 4.47) have potential therapeutic value. Enantioselective microbial reduction of a ketoester **180** and a chlorinated ketone **181** to their corresponding (*S*)-alcohols **182** and **183** was demonstrated by *P. delftensis* MY 1569 and *R. piliminae* ATCC 32762 with ees of >98% and >99%, respectively [155]. Reductions were scaled up to 23 L to produce the desired (*S*)-alcohols in 88% and 97% yields, respectively.

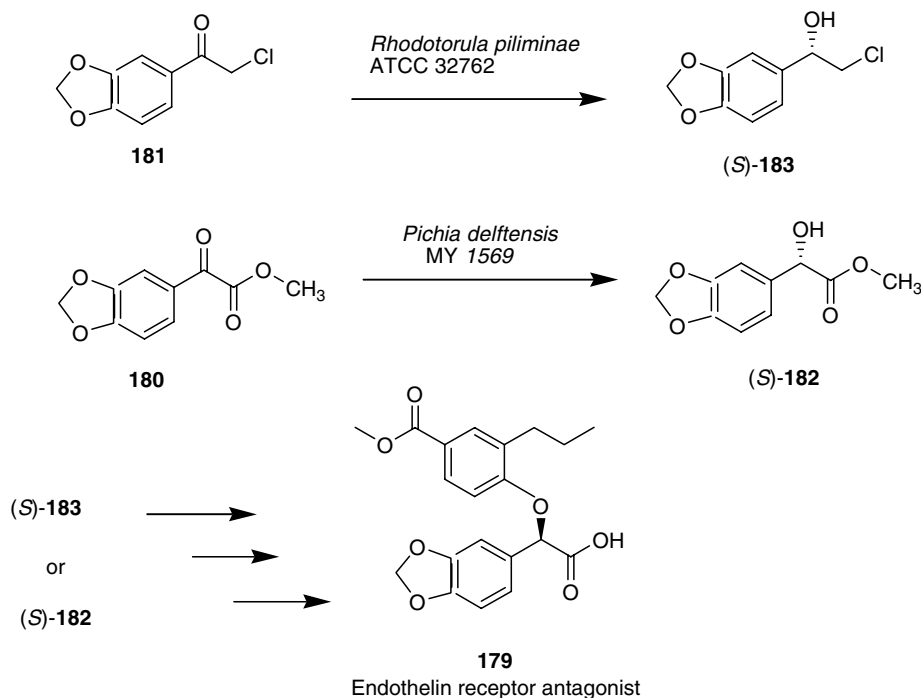


FIGURE 4.47 Preparation of a chiral synthon for an endothelin receptor antagonist **179**: enantioselective microbial reduction of ketoester **180** and chloroketone **181**.

4.22 ANTIANXIETY DRUG

4.22.1 ENZYMIC PREPARATION OF 6-HYDROXYBUSPIRONE

Buspirone (Buspar, **184**, Figure 4.48) is a drug used for the treatment of anxiety and depression that is thought to produce its effects by binding to the serotonin 5HT_{1A} receptor [156]. Mainly as a result of hydroxylation reactions, it is extensively converted to various metabolites [157], and blood concentrations return to low levels a few hours after dosing. A major metabolite, 6-hydroxybuspirone **185**, produced by the action of liver cytochrome P450 CYP3A4, is present at much higher concentrations in human blood than buspirone itself. This metabolite has anxiolytic effects in an anxiety model using rat pups and binds to the human 5HT_{1A} receptor [158]. Although the metabolite has only about a third of the affinity for the human 5HT_{1A} receptor as buspirone, it is present in human blood at 30 to 40 times higher concentration than buspirone following a dose of buspirone, and therefore may be responsible for much of the effectiveness of the drug [159]. For the development of 6-hydroxybuspirone as a potential anti-anxiety drug, preparation and testing of the two enantiomers as well as the racemate was of interest. Both the *R*- and *S*-enantiomers, isolated by chiral HPLC, were effective in tests using a rat model of anxiety [160]. Whereas the *R*-enantiomer showed somewhat tighter binding and specificity for the 5HT_{1A} receptor, the *S*-enantiomer had the advantage of being cleared more slowly from the blood. An enzymatic process was developed for resolution of 6-acetoxibuspirone **186**. L-amino acid

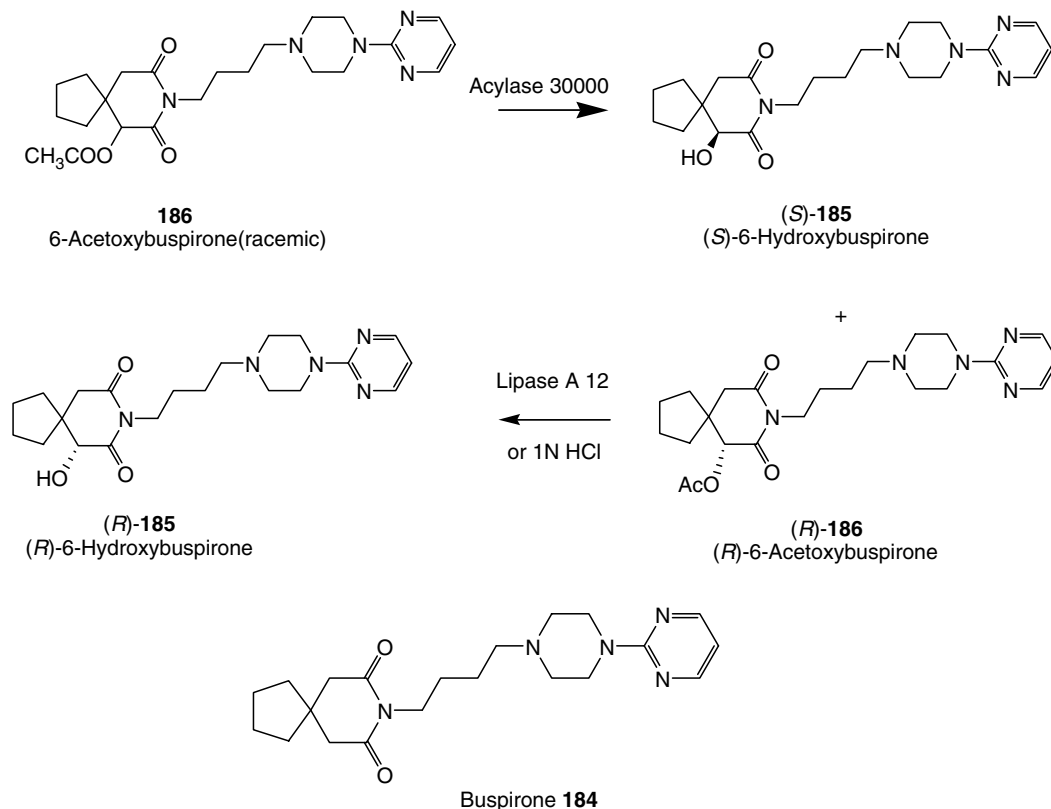


FIGURE 4.48 Lipase-catalyzed preparation of (*S*)-**185** and (*R*)-6-hydroxybuspirone **185** by a resolution process.

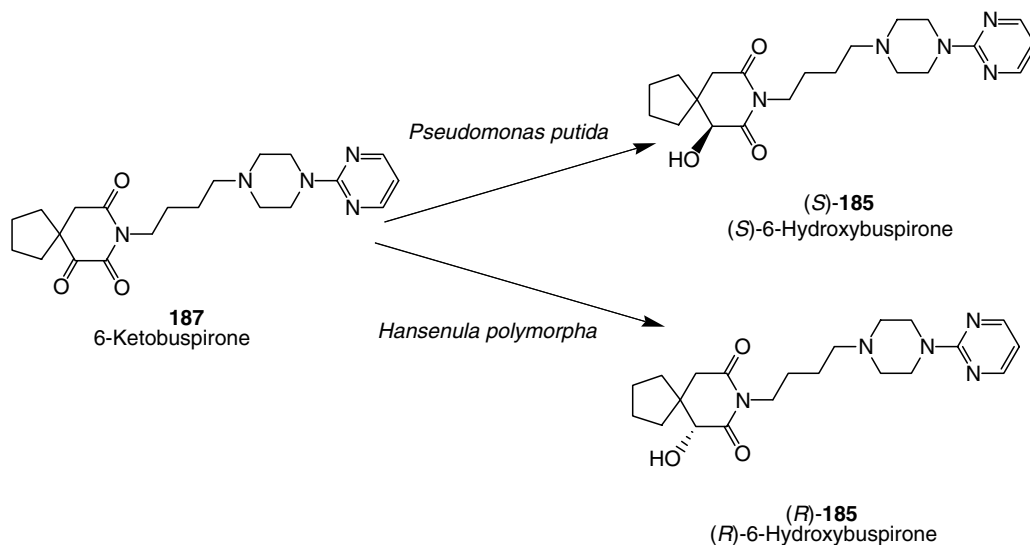


FIGURE 4.49 Enzymatic preparation of (S)-**185** and (R)-6-hydroxybuspirone **185** by reduction of 6-ketobuspirone **187**.

acylase from *Aspergillus melleus* (Amano acylase 30000) was used to hydrolyze racemic 6-acetoxibuspirone to (S)-6-hydroxybuspirone in 96% ee after 46% conversion. The remaining (R)-6-acetoxibuspirone with 84% ee was converted to (R)-6-hydroxybuspirone by acid hydrolysis. The ee of both enantiomers could be improved to >99% by crystallization as a metastable polymorph [161]. Direct hydroxylation of buspirone to (S)-6-hydroxybuspirone by *S. antibioticus* ATCC 14980 has also been described [161]. In an alternate process, enantioselective microbial reduction of 6-ketobuspirone **187** (Figure 4.49) to either (R)- or (S)-6-hydroxybuspirone was described. About 150 microorganisms were screened for the enantioselective reduction of **187**. *R. stolonifer* SC 13898, *R. stolonifer* SC 16199, *Neurospora crassa* SC 13816, *Mucor racemosus* SC 16198, and *P. putida* SC 13817 gave >50% reaction yields and >95% ees of (S)-6-hydroxybuspirone. The yeast strains *H. polymorpha* SC 13845 and *C. maltosa* SC 16112 gave (R)-6-hydroxybuspirone in >60% reaction yield and >97% ee [162].

4.23 ANTIPSYCHOTIC AGENT

4.23.1 ENZYMATIC REDUCTION OF 1-(4-FLUOROPHENYL)-4-[4-(5-FLUORO-2-PYRIMIDINYL)-1-PIPERAZINYL]-1-BUTANONE

During the past few years, much effort has been directed toward the understanding of the sigma receptor system in the brain and endocrine tissues. This effort has been motivated by the hope that the sigma site may be a target of a new class of antipsychotic drugs [163,164]. The characterization of the sigma system helped to clarify the biochemical properties of the distinct haloperidol-sensitive sigma binding site, the pharmacological effects of sigma drugs in several assay systems, and the transmitter properties of a putative endogenous ligand for the sigma site [165,166]. R-(+) compound **188** [BMV-14802] is a sigma ligand and has a high affinity for sigma binding sites and antipsychotic efficacy [167,168]. The stereoselective microbial reduction of keto compound 1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanone **189** to yield the corresponding hydroxy compound R-(+)-BMV-14802 **188** (Figure 4.50) has been developed by Patel et al. [169]. Among various microorganisms

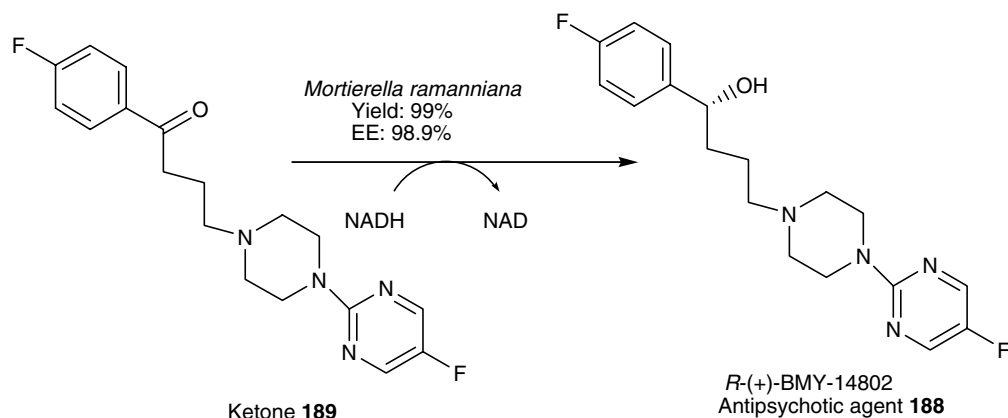


FIGURE 4.50 Enantioselective microbial reduction of **189** to prepare *R*-(+)-BMY-14802, an antipsychotic agent.

evaluated for the reduction of ketone **189**, *M. ramanniana* ATCC 38191 predominately reduced compound **189** to *R*-(+)-BMY-14802, and *Pullularia pullulans* ATCC 16623 reduced compound **189** to *S*-(-)-BMY-14802. An ee of >98% was obtained in each reaction.

In a two-stage process for the reduction of compound **189**, cells of *M. ramanniana* ATCC 38191 were grown in a 380 L fermentor, and cells harvested after 31 h growth were used for the reduction in a 15 L fermentor using 20% cell suspensions (20% w/v, wet cells). Ketone **189** was used at 2 g/L concentration and glucose was supplemented at 20 g/L concentration during the biotransformation process to generate NADH required for the reduction. After a 24 h biotransformation period, about 90% yield and 99.0% ee of *R*-(+)-BMY-14802 were obtained. The *R*-(+)-BMY-14802 was isolated from the fermentation broth in overall 70% yield, 99.5% ee, and 99% chemical purity.

A single-stage fermentation–biotransformation process was demonstrated for the reduction of ketone **189** to *R*-(+)-BMY-14802 by the cells of *M. ramanniana* ATCC 38191. Cells were grown in a 20 L fermentor containing 15 L of medium. After 40 h of growth, the biotransformation process was initiated by addition of 30 g of ketone **189** and 300 g of glucose, and was completed in 24 h, with a reaction yield of 100% and an ee of 98.9% for *R*-(+)-BMY-14802. At the end of the biotransformation process, cells were removed by filtration and product was recovered from the filtrate in overall 80% recovery.

4.24 ENZYMATIC ACYLOIN CONDENSATION

Asymmetric α -hydroxyketones (acyloins) are important classes of intermediates in organic synthesis due to their bifunctional aspect, especially having one chiral center amenable to further modification. Enzyme-mediated acyloin formation could provide an advantageous, environment-friendly method to prepare optically active asymmetric acyloins [170]. Acyloin formation mediated by yeast pyruvate decarboxylase and bacterial benzoylformate decarboxylase [171] has been reported. Although phenylpyruvate decarboxylase (PPD) [172] for decarboxylation of phenylpyruvic acid has been known for a long time, recently we reported the acyloin condensation catalyzed by PPD [173,174]. *Achromobacter eurydice* PPD was used to catalyze the asymmetric acyloin condensation of phenylpyruvate (**190**, Figure 4.51) with various aldehydes **191** to produce optically active acyloins $\text{PhCH}_2\text{COCH}(\text{OH})\text{R} **192**. The$

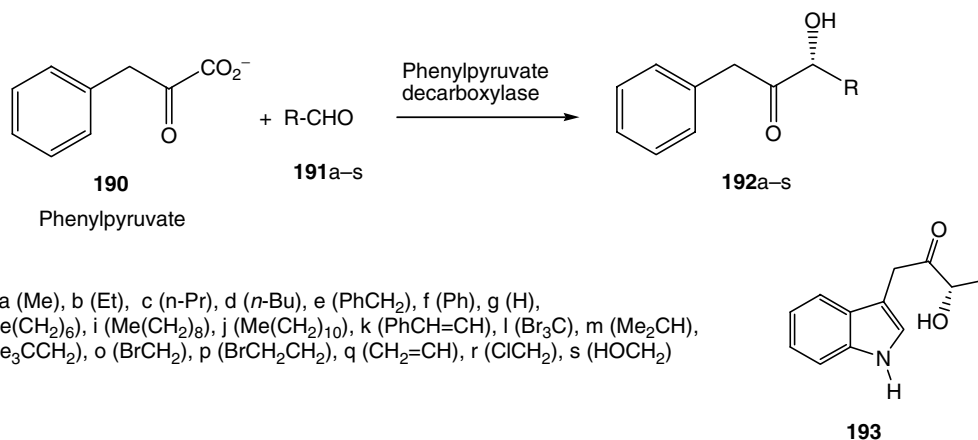


FIGURE 4.51 Phenylpyruvate decarboxylase catalyzed acyloin condensation reactions.

acyloin condensation yield decreased with increasing chain length for straight-chain aliphatic aldehydes from 76% for acetaldehyde to 24% for valeraldehyde. The ees of the acyloin products were of 87 to 98%. Low yields of acyloin products were obtained with chloroacetaldehyde (13%) and glycoaldehyde (16%). Indole-3-pyruvate was a substrate of the enzyme and provided acyloin condensation product 3-hydroxy-1-(3-indolyl)-2-butanone (**193**) with acetaldehyde in 19% yield.

4.25 ENANTIOSELECTIVE ENZYMATIC CLEAVAGE OF CARBOBENZYLOXY GROUPS

Amino groups often require protection during synthetic transformations elsewhere in the molecule; at some point, the protecting group must be removed. Enzymatic protection and deprotection under mild conditions have been demonstrated previously. Penicillin G amidase and phthalalyl amidase have been used for the enzymatic deprotection of the phenylacetyl and phthaloyl groups from the corresponding amido or imido compounds [175,176]. Acylases have been used widely in the enantioselective deprotection of *N*-acetyl-DL-amino acids [177]. Enzymatic deprotection of *N*-carbamoyl L-amino acids and *N*-carbamoyl D-amino acids has been demonstrated by microbial L-carbamoylases and D-carbamoylases, respectively [178,179].

The carbobenzyloxy (Cbz) group is commonly used to protect amino and hydroxyl groups during organic synthesis. Chemical deprotection is usually achieved by hydrogenation with a palladium catalyst [180,181]. However, during chemical deprotection some groups are reactive (e.g., carbon-carbon double bonds) under, or may interfere (e.g., thiols or sulfides) with, the hydrogenolysis conditions. An enantioselective enzymatic deprotection process has been developed that can be performed under mild conditions without damaging any otherwise susceptible groups in the molecule. A microbial culture was isolated from soil and identified as *S. paucimobilis* strain SC 16113; this culture catalyzed the enantioselective cleavage of Cbz groups (Figure 4.52) from various Cbz-protected amino acids [182]. Only Cbz-L-amino acids were deprotected, giving complete conversion to the corresponding L-amino acid. Cbz-D-amino acids gave <2% reaction yield.

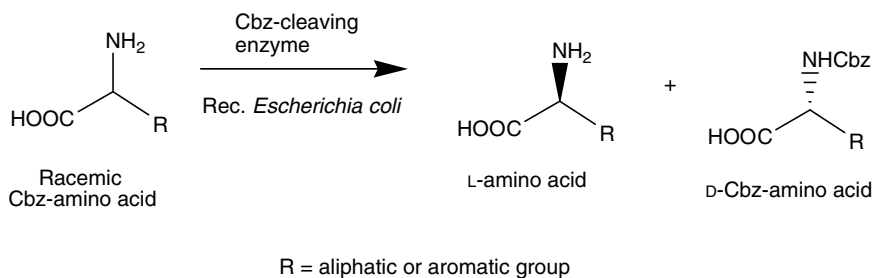


FIGURE 4.52 Enantioselective enzymatic cleavage of carbobenzyloxy (Cbz) groups from Cbz-amino acids.

Racemic Cbz-amino acids were also evaluated as substrates for hydrolysis by cell extracts of *S. paucimobilis* SC 16113. As anticipated, only the L-enantiomer was hydrolyzed, giving the L-amino acids in >48% yields and >99% ees. The unreacted Cbz-D-amino acids were recovered in >48% yield and >98% ee [182]. This enzyme has been cloned and overexpressed in *E. coli* [183].

4.26 CONCLUSION

The production of single enantiomers of drug intermediates is increasingly important in the pharmaceutical industry. Organic synthesis is one approach to the synthesis of single enantiomers, and biocatalysis provides an alternate opportunity to prepare pharmaceutically useful chiral compounds. The advantages of biocatalysis over chemical catalysis are that enzyme-catalyzed reactions are stereoselective and regioselective and can be carried out at ambient temperature and atmospheric pressure. The use of different classes of enzymes for the catalysis of many different types of chemical reactions is capable of generating a wide variety of chiral compounds. This includes the use of hydrolytic enzymes such as lipases, esterases, proteases, dehalogenases, acylases, amidases, nitrilases, lyases, epoxide hydrolases, decarboxylases, and hydantoinases in the resolution of a variety of racemic compounds and in the asymmetric synthesis of enantiomerically enriched chiral compounds. Oxido-reductases and aminotransferases have been used in the synthesis of chiral alcohols, aminoalcohols, amino acids, and amines. Aldolases and decarboxylases have been effectively used in asymmetric synthesis by aldol condensation and acyloin condensation reactions. Monooxygenases have been used in enantioselective and regioselective hydroxylation, epoxidation, and Baeyer–Villiger reactions. Dioxygenases have been used in the chemo-enzymatic synthesis of chiral diols. During the last decade, progress in biochemistry, protein chemistry, molecular cloning, and random and site-directed mutagenesis, directed evolution of biocatalysts, and fermentation technology has opened up unlimited access to a variety of enzymes and microbial cultures as tools in organic synthesis.

ACKNOWLEDGMENT

The author would like to acknowledge Ronald Hanson, Animesh Goswami, Amit Banerjee, Venkata Nanduri, Jeffrey Howell, Steven Goldeberg, Robert Johnston, Mary-Jo Donovan, Dana Cazzulino, Thomas Tully, Thomas LaPorte, Shankar Swaminathan, Lawrence Parker, John Venit, John Wasyluk, Michael Montana, Ronald Eiring, Sushil Srivatava, Ambraish Singh, Rapheal Ko, Linda Chu, Clyde McNamee, John Thottathil, David Kronenthal, and Richard Mueller for research collaboration and Dr. Richard Mueller for reviewing this manuscript and making valuable suggestions.

REFERENCES

1. Food & Drug Administration, FDA's statement for the development of new stereoisomeric drugs, *Chirality*, 4, 338, 1992.
2. Buckland, B., Robinson D., and Chartrain, M., Biocatalysis for pharmaceuticals: status and prospects for a key technology, *Metabol. Eng.*, 2, 42, 2000.
3. Pesti, J.A. and DiCosimo, R., Recent progress in enzymatic resolution and desymmetrization of pharmaceuticals and their intermediates, *Curr. Opin. Drug Discovery Dev.*, 6, 884, 2003.
4. Patel, R.N., Biocatalytic synthesis of chiral pharmaceutical intermediates, *Food Technol. Biotechnol.*, 42, 305, 2004.
5. Faber, K. and Kroutil, W., New enzymes for biotransformations, *Curr. Opin. Chem. Biol.*, 9(2), 181, 2005.
6. Sheldon, R.A. et al., Biocatalysis in ionic liquids, *Green Chem.*, 4(2), 147, 2002.
7. Turner, N.J., Enzyme catalyzed deracemization and dynamic kinetic resolution reactions, *Curr. Opin. Chem. Biol.*, 8(2), 114, 2004.
8. Mahmoudian, M., Development of bioprocesses for the generation of anti-inflammatory, antiviral and anti-leukaemic agents, *Focus Biotechnol.*, 1(Novel Frontiers in the Production of Compounds for Biomedical Use), 249, 2001.
9. Zaks, A., Industrial biocatalysis, *Curr. Opin. Chem. Biol.*, 5(2), 130, 2001.
10. Patel, R.N., Stereoselective biocatalysis for synthesis of some chiral pharmaceutical intermediates, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, pp. 87–130.
11. Barrish, J. et al., Amino diol HIV-protease inhibitors, 1: design, synthesis, and preliminary SAR, *J. Med. Chem.*, 37, 1758, 1994.
12. Patel, R.N. et al., Preparation of chiral synthon for HIV-protease inhibitor: stereoselective microbial reduction of *N*-protected α -aminochloroketone, *Tetrahedron Asymmetry*, 8, 2547, 1997.
13. Bold, G. et al., New aza-dipeptide analogs as potent and orally absorbed HIV-1 protease inhibitors: candidates for clinical development, *J. Med. Chem.*, 41, 3387, 1998.
14. Robinson, B. et al., BMS-232632, a highly potent human immunodeficiency virus protease inhibitor that can be used in combination with other available antiretroviral agents, *Antimicrob. Agents Chemother.*, 44, 2093, 2000.
15. Patel, R.N., Chu, L., and Mueller, R., Diastereoselective microbial reduction of (*S*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester, *Tetrahedron Asymmetry*, 14, 3105, 2003.
16. Xu, Z. et al., Process research and development for an efficient synthesis of the HIV-protease inhibitor BMS-232632, *Organic Process R&D*, 6, 323, 2002.
17. Ireland, C., Leeson, P., and Castaner, J., Lobucavir: antiviral, *Drugs Future*, 22, 359, 1997.
18. Hanson, R. et al., Regioselective enzymatic aminoacylation of lobucavir to give an intermediate for lobucavir prodrug, *Bioorg. Med. Chem.*, 8, 2681, 2000.
19. Chartrain, M. et al., Bioconversion of indene to *cis*-(1*S*,2*R*)-indandiol and *trans*-(1*R*,2*R*)-indandiol by *Rhodococcus* sp., *J. Ferment Technol.*, 86, 550, 1998.
20. Buckland, B.C., Robinson, D.K., and Chartrain, M., Biocatalysis for pharmaceuticals: status and prospects for a key technology, *Met. Eng.*, 2, 42, 2000.
21. Chartrain, M. et al., Metabolic engineering and directed evolution for the production of pharmaceuticals, *Curr. Opin. Biotechnol.*, 11, 209, 2000.
22. Zhang, N. et al., Directed evolution of toluene dioxygenase from *Pseudomonas putida* for improved selectivity toward *cis*-(1*S*,2*R*)-indandiol during indane bioconversion, *Metabol. Eng.*, 2, 339, 2000.
23. Amanullah, A. et al., Measurement of strain-dependent toxicity in the indene bioconversion using multiparameter flow cytometry, *Biotechnol. Bioeng.*, 80, 239, 2002.
24. Zhang, J. et al., Bioconversion of indene to *trans*-2*S*,1*S*-bromoindanol and 1*S*,2*R*-indene oxide by a bromoperoxidase/dehydrogenase preparation from *Curvularia protuberata* MF5400, *J. Ferment. Bioeng.*, 80, 244, 1995.
25. Holton, R., Biediger, R., and Joatman, P., Semisynthesis of taxol and taxotere, in *Taxol: Science and Application*, Suffness, M., Ed., CRC Press, New York, 1995.

26. Kingston, D., Natural taxoids: structure and chemistry, in *Taxol: Science and Application*, Suffness, M., Ed., CRC Press, New York, 1995.
27. Baldini, E. et al., Multicenter randomized phase III trial of Epirubicin plus Paclitaxel vs. Epirubicin followed by Paclitaxel in metastatic breast cancer patients: focus on cardiac safety, *Br. J. Cancer*, 91, 45, 2004.
28. Patel R.N., Tour de paclitaxel: biocatalysis for semisynthesis, *Ann. Rev. Microbiol.*, 98, 361, 1995.
29. Hanson R.L. et al., Site-specific enzymatic hydrolysis of taxanes at C-10 and C-13, *J. Biol. Chem.*, 269, 22145, 1994.
30. Nanduri, B. et al., Fermentation and isolation of C-10 deacetylase for the production of 10-DAB from baccatin III, *Biotech. Bioeng.*, 48, 547, 1995.
31. Hanson, R. et al., Enzymic hydrolysis of 7-xylosyltaxanes by xylosidase from *Moraxella* sp., *Biotechnol. Appl. Biochem.*, 26, 153, 1997.
32. Patel, R.N. et al., Stereoselective microbial reduction of 2-keto-3-(*N*-benzoylamino)-3-phenyl propionic acid ethyl ester: synthesis of taxol side-chain synthon, *Tetrahedron Asymmetry*, 4, 2069, 1993.
33. Patel, R.N. et al., Enzymic preparation of (3*R*-*cis*)-3-(acetyloxy)-4-phenyl-2-azetidinone: a taxol side-chain synthon, *Biotech. Appl. Biochem.*, 20, 23, 1994.
34. Baloglu, E. and Kingston, D., The taxane diterpenoids, *J. Nat. Prod.*, 62, 1068, 1999.
35. Rose, W., Preclinical pharmacology of BMS-275183, an orally active taxane, *Clin. Cancer Res.*, 7, 2016, 2001.
36. Holton, R., Nadizadeh, H., and Beidiger, R., Preparation of substituted taxanes as antitumor agents, Eur. Patent Appl. 17 pp. EP 534708 A1 19930331 CAN 119:49693 AN 1993:449693, 1993.
37. Patel, R.N. et al., Enzymatic preparation of (3*R*)-*cis*-3-acetyloxy-4-(1,1-dimethylethyl)-2-azetidinone: a side-chain synthon for an orally active taxane, *Tetrahedron Asymmetry*, 14, 3673, 2003.
38. Hanson, R. and Patel, R., Process for the preparation of C-4 deacetyltaxanes, PCT Int. Appl., WO 2000010989 A1 20000302 CAN 132:194525 AN 2000:144872, 2000.
39. Goodin, S., Kane, M.P., and Rubin, E.H., Epothilones: mechanism of action and biologic activity, *J. Clin. Oncol.*, 22(10), 2015, 2004.
40. Nicolaou, K.C., Roschangar, F., and Vourloumis, D., Chemical biology of epothilones, *Angewandte Chemie*, International Edition, 37(15), 2014, 1998.
41. Altmann, K-H., The merger of natural product synthesis and medicinal chemistry: on the chemistry and chemical biology of epothilones, *Org. Biomol. Chem.*, 2(15), 2137, 2004.
42. Mulzer, J., Mantoulidis, A., and Ohler, E., Total syntheses of epothilones B and D, *J. Org. Chem.*, 65(22), 7456, 2000.
43. Altmann, K-H. et al., The natural products epothilones A and B as lead structures for anticancer drug discovery: chemistry, biology, and SAR studies, *Progr. Med. Chem.*, 42, 171, 2004.
44. Lin, N., Brakora, K., and Seiden, M., BMS-247550 (Bristol-Myers Squibb/GBF), *Curr. Opin. Investig. Drugs* (Thomson Current Drugs), 4(6), 746, 2003.
45. Gerth, K. et al., Studies on the biosynthesis of epothilones: hydroxylation of epo A and B to epothilones E and F, *J. Antibiot.*, 55(1), 41, 2002.
46. Li, W. et al., Microbial transformation method for the preparation of an epothilone, PCT Int. Appl., WO 2000039276 A2 20000706 CAN 133:88294 AN 2000:457191, 2000.
47. Basch, J.D. et al., The epothilone B hydroxylase and ferredoxin genes of *Amycolatopsis* and their use in the development of strains for the manufacture of hydroxylated epothilones, PCT Int. Appl., WO 2004078978 A1 20040916 CAN 141:255532 AN 2004:756881, 2004.
48. Umezawa, H. et al., Structure of an antitumor antibiotic, spergualin, *J. Antibiot.*, 34, 1622, 1981.
49. Umeda, Y. et al., Synthesis and antitumor activity of spergualin analogues, III: novel method for synthesis of optically active 15-deoxyspergualin and 15-deoxy-11-*O*-methylspergualin, *J. Antibiot.*, 40, 1316, 1987.
50. Patel, R.N., Banerjee, A., and Szarka, L.J., Stereoselective acetylation of racemic 7-[*N,N*-bis-benzyloxycarbonyl]-*N*-(guanidinoheptanoyl)]- α -hydroxyglycine, *Tetrahedron Asymmetry*, 8, 1767, 1997.
51. Nemoto, K. et al., Effect of 15-deoxyspergualin on graft-v-host disease in mice, *Trans. Proc.*, 19(5), 3985, 1987.

52. Carboni, J.M., Hurlburt, W.W., and Gottardis, M.M., Synergistic methods and compositions using IGF-1 receptor inhibitors and EGF receptor inhibitors for treating cancer, PCT Int. Appl., WO 2004030625 A2 20040415 CAN 140:315050 AN 2004:308360, 2004.
53. Beaulieu, F. et al., Preparation of isopurines and related compounds as tyrosine kinase inhibitors for the treatment of cancer, PCT Int. Appl., WO 2004063151 A2 20040729 CAN 141:140461 AN 2004:606437, 2004.
54. Hanson, R.L. et al., Purification and cloning of a ketoreductase used for the preparation of chiral alcohols, *Adv. Synth. Cat.*, 347(7&8), 1073, 2005.
55. Ondetti, M.A. and Cushman, D.W., Inhibition of renin-angiotensin system: a new approach to the theory of hypertension, *J. Med. Chem.*, 24, 355, 1981.
56. Ondetti, M.A., Rubin, B., and Cushman, D.W., Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents, *Science*, 196, 441, 1977.
57. Cushman, D.W. and Ondetti, M.A., Inhibitors of angiotensin-converting enzyme for treatment of hypertension, *Biochem. Pharm.*, 29, 1871, 1980.
58. Goodhue, C.T. and Schaeffer, J.R., Preparation of L-(+)- β -hydroxyisobutyric acid by bacetrial oxidation of isobutyric acid, *Biotechnol. Bioeng.*, 13, 203, 1971.
59. Patel, R.N. et al., Stereoselective enzymatic hydrolysis of α -[acetylthio)methyl]benzenepropanoic acid and 3-acetylthio-2-methylpropanoic acid, *Biotechnol. Appl. Biochem.*, 16, 34, 1992.
60. Patel, R.N. et al., Stereoselective enzymatic esterification of 3-benzoylthio-2-methylpropanoic acid, *Appl. Microbiol. Biotechnol.*, 36, 29, 1991.
61. Moniot, J.L., Preparation of N-[2-(mercaptomethyl)propionyl]-L-prolines, U.S. Patent Appl., CN 88-100862, 1988.
62. Ondetti, M.A., Miguel, A., and Krapcho, J., Mercaptoacyl derivatives of substituted prolines, U.S. Patent, 4316906, 1982.
63. Patel, R.N., Robison, R.S., and Szarka, L.J., Stereoselective enzymic hydrolysis of 2-cyclohexyl- and 2-phenyl-1,3-propanediol diacetate in biphasic systems, *Appl. Microbiol. Biotechnol.*, 34, 10, 1990.
64. Karenewsky, D.S. et al., (Phosphinyloxy)acyl amino acid inhibitors of angiotensin converting enzyme (ACE), 1: discovery of (S)-1-[6-amino-2-[[hydroxy(4-phenylbutyl)phosphinyl]oxy]-1-oxohexyl]-L-proline, a novel orally active inhibitor of ACE, *J. Med. Chem.*, 31, 204, 1988.
65. Hanson, R.L. et al., Transformation of N- ϵ -CBZ-L-lysine to CBZ-L-oxylysine using L-amino acid oxidase from *Providencia alcalifaciens* and L-2-hydroxy-isocaproate dehydrogenase from *Lactobacillus confusus*, *Appl. Microbiol. Biotechnol.*, 37, 599, 1992.
66. Delaney, N.G. et al., Amino acid and peptide derivatives as inhibitors of neutral endopeptidase and their use as antihypertensives and diuretics, Eur. Patent, EP361365, 1988.
67. Robl, J.A. et al., Dual metalloprotease inhibitors: mercaptoacetyl-based fused heterocyclic dipeptide mimetics as inhibitors of angiotensin-converting enzyme and neutral endopeptidase, *J. Med. Chem.*, 40, 1570, 1997.
68. Seymour, A.A., Swerdel, J.N., and Abboa-Offei, B.E., Antihypertensive activity during inhibition of neutral endopeptidase and angiotensin converting enzyme, *J. Cardiovasc. Pharmacol.*, 17, 456, 1991.
69. Wichman, R. et al., Continuous enzymic transformation in an enzyme membrane reactor with simultaneous NAD(H) regeneration, *Biotechnol. Bioeng.*, 23, 2789, 1981.
70. Kragl, U., Vasic-Racki, D., and Wandrey, C., Continuous processes with soluble enzymes, *Chem. Ing. Tech.*, 64, 499, 1992.
71. Hanson, R.L. et al., Enzymatic synthesis of L-6-hydroxynorleucine, *Bioorg. Med. Chem.*, 7, 2247, 1999.
72. Patel, R.N., Enzymatic synthesis of chiral intermediates for Omapatrilat, an antihypertensive drug, *Biomol. Eng.*, 17(6), 167, 2001.
73. Rumero, A. et al., Chemical synthesis of allysine ethylene acetal and conversion *in situ* into 1-piperidine-6-carboxylic acid: key intermediate of the α -aminoadipic acid for β -lactam antibiotics biosynthesis, *Bioorg. Med. Chem.*, 3, 1237, 1995.
74. Hanson, R. et al., Enzymatic synthesis of allysine ethylene acetal, *Enzyme Microb. Technol.*, 26, 348, 2000.

75. Schütte, H. et al., Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidinii*, *Eur. J. Biochem.*, 62, 151, 1976.
76. Hou, C.T. et al., NAD-linked formate dehydrogenase from methanol-grown *Pichia pastoris* NRRL-Y-7556, *Arch. Biochem. Biophys.*, 216, 296, 1982.
77. Patel, R.N., Biocatalytic preparation of a chiral synthon for a vasoepitidase inhibitor: enzymatic conversion of *N*2-[*N*-[(phenylmethoxy)carbonyl]L-homocysteiny]L-lysine (1>1')-disulfide to [4*S*-(4*a*,7*a*,10*ab*)]1-octahydro-5-oxo-4-[(phenylmethoxy) carbonyl]amino]-7*H*-pyrido-[2,1-*b*][1,3]thiazepin-7-carboxylic acid methyl ester by a novel L-lysine ϵ -aminotransferase, *Enzyme Microb. Technol.*, 27, 376, 2000.
78. Ward, O. and Young, C., Reductive biotransformations of organic compounds by cells or enzymes of yeast, *Enzyme Microb. Technol.*, 12, 482, 1990.
79. Csuk, R. and Glanzer, B., Yeast mediated stereoselective biocatalysis, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000.
80. Patel, R.N. et al., Stereoselective reduction of β -ketoesters by *Geotrichum candidum*, *Enzyme Microb. Technol.*, 14, 731, 1992.
81. Matsuyama, A., Yamamoto, H., and Kobayashi, Y., Practical application of recombinant whole-cell biocatalysts for the manufacturing of pharmaceutical intermediates such as chiral alcohols, *Org. Process R&D*, 6, 558, 2002.
82. Davis, S. et al., Halohydrin dehalogenases and method for production of 4-cyano-3-hydroxybutyric acid esters and amides, PCT Int. Appl., WO 2004015132, A2 20040219, CAN 140:198191, AN 2004:143313, 2004.
83. Fishman, A. et al., Enzymatic resolution as a convenient method for the production of chiral resolved materials on an industrial scale, *Biotechnol. Bioeng.*, 74, 256, 2001.
84. Sit, S. et al., Synthesis, biological profile, and quantitative structure–activity relationship of a series of novel 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, *J. Med. Chem.*, 33, 2982, 1990.
85. Patel, R.N. et al., Enantioselective microbial reduction of 3,5-dioxo-6-(benzyloxy)hexanoic acid, ethyl ester, *Enzyme Microb. Technol.*, 15, 1014, 1993.
86. Patel, R.N., McNamee, C., and Szarka, L., Enantioselective enzymic acetylation of racemic [4-[4 α ,6 β (*E*)]-6-[4,4-bis(4-fluorophenyl)-3-(1-methyl-1*H*-tetrazol-5-yl)-1,3-butadienyl]tetrahydro-4-hydroxy-2*H*-pyran-2-one, *Appl. Microbiol. Biotechnol.*, 38, 56, 1992.
87. Gijzen, H. and Wong, C-H., Sequential three- and four-substrate aldol reactions catalyzed by aldolases, *J. Amer. Chem. Soc.*, 116, 8422, 1994.
88. Greenberg, W. et al., Development of an efficient, scalable, aldolase-catalyzed process for enantioselective synthesis of statin intermediates, *Proc. Natl. Acad. Sci. USA*, 101, 5788, 2004.
89. Steiger, A., Pyun, H., and Coates, R., Synthesis and characterization of aza analog inhibitors of squalene and geranylgeranyl diphosphate synthases, *J. Org. Chem.*, 57, 3444, 1992.
90. Lawrence, M., Biller, S., and Fryszman, O., Preparation of α -phosphonosulfonic squalene synthetase inhibitors, U.S. Patent 5447922, 1995.
91. Patel, R.N., Banerjee, A., and Szarka, L., Stereoselective acetylation of [1-(hydroxy)-4-(3-phenoxyphenyl)butyl]phosphonic acid diethyl ester, *Tetrahedron Asymmetry*, 8, 1055, 1997.
92. Nakane, M., Preparation and formulation of 7-oxabicycloheptane substituted sulfonamide prostaglandin analogs useful in treatment of thrombotic disease, U.S. Patent 4,663,336, 1987.
93. Ford-Hutchinson, A.W., Innovations in drug research: inhibitors of thromboxane and leukotrienes, *Clin. Exper. Allergy*, 21, 272, 1991.
94. Das, J. et al., Synthesis of optically 7-oxabicyclo[2.2.1]heptanes and assignment of absolute configuration, *Synthesis*, 12, 1100, 1987.
95. Patel, R.N. et al., Stereoselective microbial/enzymatic oxidation of (exo,exo)-7-oxabicyclo[2.2.1]-heptane-2,3-dimethanol to the corresponding chiral lactol and lactone, *Enzyme Microb. Technol.*, 14, 778, 1992.
96. Patel, R.N. et al., Stereoselective enzymic hydrolysis of (exo,exo)-7-oxabicyclo[2.2.1]heptane-2,3-dimethanol diacetate ester in a biphasic system, *Appl. Microbiol. Biotechnol.*, 37, 180, 1992.
97. Chaffman, M. and Brogden, R.N., Diltiazem: a review of its pharmacological properties and therapeutic efficacy, *Drugs*, 29, 387, 1985.

98. Kawai, C. et al., Comparative effects of three calcium antagonists, diltiazem, verapamil and nifedipine, on the sinoatrial and atrioventricular nodes, *Exp. Cir.*, 63, 1035, 1981.
99. Isshiki, T., Pegram, B., and Frohlich, E., Immediate and prolonged hemodynamic effects of TA-3090 on spontaneously hypertensive (SHR) and normal Wistar-Kyoto (WKY) rats, *Cardiovasc. Drug Ther.*, 2, 539, 1988.
100. Floyd, D.M. et al., Synthesis of benzazepinone and 3-methylbenzothiazepinone analogs of diltiazem, *J. Org. Chem.*, 55, 5572, 1990.
101. Patel, R.N. et al., Stereospecific microbial reduction of 4,5-dihydro-4-(4-methoxyphenyl)-6-(trifluoromethyl-1H-1)-benzazepin-2-one, *Enzyme Microb. Technol.*, 13, 906, 1991.
102. Edwards, G. and Weston, A.H., Structure-activity relationships of K⁺ channel openers, *Trends Pharmacol. Sci.* 11, 417-422, 1990.
103. Robertson, D.W. and Steinberg, M.I., Potassium channel openers: new biological probes, *Ann. Med. Chem.*, 24, 91, 1989.
104. Hamilton, T.C. and Weston, A.H., Cromakalim, nicorandil and pinacidil: novel drugs which open potassium channels in smooth muscle, *Gen. Pharmac.*, 20, 1, 1989.
105. Ashwood, V.A. et al., Synthesis and antihypertensive activity of 4-(cyclic amido)-2H-1-benzopyrans, *J. Med. Chem.*, 29, 2194, 1986.
106. Bergmann, R., Eiermann, V., and Gericke, R.J., 4-Heterocycloxy-2H-1-benzopyran potassium channel activators, *J. Med. Chem.*, 33, 2759, 1990.
107. Patel, R.N. et al., Stereoselective epoxidation of 2,2-dimethyl-2H-1-benzopyran-6-carbonitrile, *Bioorg. Med. Chem.*, 2, 535, 1994.
108. Patel, R.N. et al., Stereoselective acetylation of 3,4-dihydro-3,4-dihydroxy-2,2-dimethyl-2H-1-benzopyran-6-carbonitrile, *Tetrahedron Asymmetry*, 6, 123, 1995.
109. Archelas, A. and Furstoss, R., Biocatalytic approaches for the synthesis of enantiopure epoxides, *Top. Curr. Chem.*, 200, 159, 1999.
110. Mischitz, M. et al., Asymmetric microbial hydrolysis of epoxides, *Tetrahedron Asymmetry*, 6, 1261, 1995.
111. Catt, J.D. et al., Preparation of benzofuran and dihydrobenzofuran melatonergic agents, U.S. 5856529, CAN 130:110151, 1999.
112. Goswami, A. et al., Stereospecific enzymatic hydrolysis of racemic epoxide: a process for making chiral epoxide, *Tetrahedron Asymmetry*, 10, 3167, 1999.
113. Stecher, H. and Faber, K., Biocatalytic deracemization techniques: dynamic resolutions and stereoinversions, *Synthesis*, 1, 1, 1997.
114. Fantin, G. et al., Kinetic resolution of racemic secondary alcohols via oxidation with *Yarrowia lipolytica* strains, *Tetrahedron Asymmetry*, 11, 2367, 2000.
115. Goswami, A., Mirfakhrae, K.D., and Patel, R.N., Deracemization of racemic 1,2-diol by biocatalytic stereoinversion, *Tetrahedron Asymmetry*, 10(21), 4239, 1999.
116. Arch, J.R.S., β 3-adrenoceptors and other putative atypical β -adrenoceptors, *Pharmacol. Rev. Commun.*, 9, 141, 1997.
117. Bloom, J.D. et al., Disodium (*R,R*)-5-[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate: a potent β -adrenergic agonist virtually specific for β -3-receptors, *J. Med. Chem.*, 35, 3081, 1989.
118. Fisher, L.G. et al., BMS-187257, a potent, selective, and novel heterocyclic β -3 adrenergic receptor agonist, *Bioorg. Med. Chem. Lett.*, 6, 2253, 1994.
119. Patel, R.N. et al., Microbial synthesis of chiral intermediates for β -3-receptor agonists, *J. Am. Oil Chem. Soc.*, 75, 1473, 1998.
120. Campos, F., Bosch, M., and Guerrero, A., An efficient enantioselective synthesis of (*R,R*)-formoterol, a potent bronchodilator, using lipases, *Tetrahedron Asymmetry*, 11, 2705, 2000.
121. Bisacchi, G., Preparation of amidino and guanidino azetidinone compounds as tryptase inhibitors, WO 9967215, A1 19991229, CAN 132:64103, AN 1999:819347, 1999.
122. Wirz, B. and Walther, W., Enzymic preparation of chiral 3-(hydroxymethyl)piperidine derivatives, *Tetrahedron Asymmetry*, 3, 1049, 1992.
123. Goswami, A. et al., Chemical and enzymatic resolution of (*R,S*)-*N*-(*tert*-butoxycarbonyl)-3-hydroxymethylpiperidine, *Organic Process R&D*, 5, 415, 2001.

124. Schenk, D., Games, D., and Seubert, P., Potential treatment opportunities for Alzheimer's disease through inhibition of secretases and A β immunization, *J. Mol. Neurosci.*, 17, 259, 2001.
125. Audia, J. et al., Preparation of *N*-(phenylacetyl)di- and tripeptide derivatives for inhibiting β -amyloid peptide release, PCT Int. Appl., WO 9822494, A2 19980528, CAN 129:41414, 126.
126. Patel, R.N. et al., Enzymatic resolution of racemic secondary alcohols by lipase B from *Candida antarctica*, *J. Am. Oil Chem. Soc.*, 77, 1015, 2000.
127. Nanduri, V., Purification of a stereospecific 2-ketoreductase from *Gluconobacter oxydans*, *J Ind. Microbiol. Biotechnol.*, 25, 171, 2000.
128. Nanduri, V.B. et al., Biochemical approaches to the synthesis of ethyl 5-(*S*)-hydroxyl hexanoate and 5-(*S*)-hydroxyhexanenitrile, *Enzyme Microb. Technol.*, 28(7&8), 632, 2001.
129. Patel, R.N., Enantioselective microbial reduction of substituted acetophenones, *Tetrahedron Asymmetry*, 15, 1247, 2004.
130. Kagechika, H. et al., Retinobenzoic acids, 1: structure-activity relationships of aromatic amides with retinoidal activity, *J. Med. Chem.*, 31, 2182, 1988.
131. Shudo, K. and Kagechika, H., Structure-activity relationships of a new series of synthetic retinoids, in *Chemistry and Biology of Synthetic Retinoids*, Dawson, M.I. and Okamura, W.H., Eds., CRC Press, Boca Raton, FL, 1989.
132. Moon, R.C. and Itri, L.M., Retinoids and cancer, *Retinoids*, 2, 327, 1984.
133. Kagechika, H. et al., Retinobenzoic acids, 1: structure-activity relationships of aromatic amides with retinoidal activity, *J. Med. Chem.*, 31, 2182, 1988.
134. Belema, M., Zusi, F.C., and Tramposch, K.M., Active enantiomer of RAR γ -specific agonist, PCT Int. Appl., WO 0016769 A1 20000330 CAN 132:246379 AN 2000:209902, 2000.
135. Patel, R.N. et al., Enantioselective microbial reduction of 2-oxo-2-(1',2',3',4'-tetrahydro-1',1',4',4'-tetramethyl-6'-naphthalenyl)acetic acid and its ethyl ester, *Tetrahedron Asymmetry*, 13, 349, 2002.
136. Hoegenauer, G., Mechanism of action of antibacterial agents: tiamulin and pleuromutilin, *Antibiotics*, 5, 344, 1979.
137. Berner, H. et al., Chemistry of pleuromutilin, IV: synthesis of 14-*O*-acetyl-8 α -hydroxymutilin, *Tetrahedron*, 39, 1317, 1983.
138. Hanson, R.L. et al., Hydroxylation of mutilin by *Streptomyces griseus* and *Cunninghamella echinulata*, *Organic Process R&D*, 6, 482, 2002.
139. Springer, D.M. et al., Synthesis and activity of a C-8 ketopluromutilin derivative, *Bioorg. Med. Chem. Lett.*, 13, 1751, 2003.
140. Iwata, H., Tanaka, R., and Ishiguro, M., Structures of the alkaline hydrolysis products of penem antibiotic, SUN5555, *J. Antibiot.* 43, 901, 1990.
141. Matsuyama, A., Yamamoto, H., and Kobayashi, Y., Practical application of recombinant whole-cell biocatalysts for the manufacturing of pharmaceutical intermediates such as chiral alcohols, *Organic Process R&D*, 6, 558, 2002.
142. O'Donnell, M.J., Bennett, D.W., and Wu, S., The stereoselective synthesis of α -amino acids by phase-transfer catalysis, *J. Am. Chem. Soc.*, 111, 2353, 1989.
143. Schmidt U. et al., Amino acids and peptides, 71: total synthesis of the didemnins, III: synthesis of protected (2*R*,3*S*)-alloisoleucine and (3*S*,4*R*,5*S*)-isostatine derivatives—amino acids from hydroxy acids, *Synthesis*, 28, 256, 1989.
144. Bold, G., Duthaler, R.O., and Riediker, M., Enantioselective synthesis with titanium carbohydrate complexes, Part 3: enantioselective synthesis of (D)-threo- β -hydroxy- α -amino acids with titanium carbohydrate complexes, *Angew. Chem. Int. Ed. Engl.*, 28, 497, 1989.
145. Gordon, E.M. et al., *O*-Sulfated β -lactam hydroxamic acids (monosulfactams): novel monocyclic β -lactam antibiotics of synthetic origin, *J. Amer. Chem. Soc.*, 104, 6053, 1982.
146. Godfrey, J.D., Mueller, R.H., and van Langen, D.J., β -Lactam synthesis: cyclization versus 1,2-acyl migration-cyclization: the mechanism of the 1,2-acyl migration-cyclization, *Tetrahedron Lett.*, 27, 2793, 1986.
147. Hanson, R.L. et al., Synthesis of L- β -hydroxyvaline from α -keto- β -hydroxyisovalerate using leucine dehydrogenase from *Bacillus* species, *Bioorg. Chem.*, 18, 116, 1990.
148. Langer, S.Z. and Hicks, P.E., Alpha-adrenoceptor subtypes in blood vessels: physiology and pharmacology, *J. Cardiovasc. Pharmacol.*, 6, 547, 1984.

149. Manoury, P.M. et al., Synthesis and antihypertensive activity of a series of 4-amino-6,7-dimethoxy-quinoxaline derivatives, *J. Med. Chem.*, 29, 19, 1986.
150. Manoury, P., Binet, J., and Cavero, I., Pyridine derivatives and their use in therapeutic, Eur. Pat. Appl., EP 61379 A1 19820929 CAN 98:89179 AN 1983:89179, 1982.
151. Sanchez, V.M., Rebolledo, F., and Gotor, V., *Candida antarctica* lipase catalyzed resolution of ethyl (\pm)-3-aminobutyrate, *Tetrahedron Asymmetry*, 8, 37–40, 1997.
152. Baldessari, A. and Mangone, C.P., One-pot biocatalyzed preparation of substituted amides as intermediates of pharmaceuticals, *J. Mol. Catalysis B: Enzymatic.*, 11, 335, 2001.
153. Fukuroda, T. and Nishikibe, M., Enhancement of pulmonary artery contraction induced by endothelin- β -receptor antagonism, *J. Cardiovasc. Pharmacol.*, 31(Suppl. 1, Endothelin V), S169–S171, 1989.
154. Sumner, M.J. et al., Endothelin ETA and ETB receptors mediate vascular smooth muscle contraction, *Br. J. Pharmacol.*, 107, 858, 1992.
155. Krulewicz, B. et al., Asymmetric biosynthesis of key aromatic intermediates in the synthesis of an endothelin receptor antagonist, *Biocatalysis Biotransform.*, 19, 267, 2001.
156. Jajoo, H.K. et al., Metabolism of the antianxiety drug buspirone in human subjects, *Drug Met. Disposition*, 17(6), 634, 1989.
157. Mayol, R.F., Buspirone metabolite for the alleviation of anxiety, Cont.-in-part of U.S. Ser. No. 484,161 US 6150365 A 20001121 CAN 133:359250 AN 2000:819478, 2000.
158. Yevich, J.P. et al., Antianxiety composition using BMY 28674, and preparation thereof, U.S. Pat. Appl. Publ., Cont.-in-part of U.S. Ser. No. 588,220. U.S. 2003069251 A1 20030410 CAN 138:281137 AN 2003:282120, 2003.
159. Yevich, J.P. et al., S-6-Hydroxy-buspirone for treatment of anxiety, depression and related disorders, U.S. Pat. Appl. Publ., 2003.
160. Yevich, J.P. et al., R-6-hydroxybuspirone for the treatment of anxiety, depression, and other psychogenic disorders. PCT Int. Appl., WO 2003009851 A1 20030206 CAN 138:131155 AN 2003:97305, 2003.
161. Hanson, R.L. et al., Preparation of (R)- and (S)-hydroxybuspirone by enzymatic resolution and hydroxylation, *Tetrahedron Asymmetry*, 16(16), 2711, 2005.
162. Patel, R.N. et al., Enantioselective microbial reduction of 6-oxo-8-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-8-azaspiro[4.5]decane-7,9-dione, *Tetrahedron Asymmetry*, 16(16), 2778, 2005.
163. Ferris, C.D. et al., ³H]opipramol labels a novel binding site and sigma receptors in rat brain membranes, *J. Neurochem.*, 57, 729, 1991.
164. Junien, J.L. and Leonard, B.E., Drugs acting on sigma and phencyclidine receptors: a review of their nature, function, and possible therapeutic importance, *Clin. Neuropharmacol.*, 12, 374, 1989.
165. Steinfels, G.F., Tam, S.W., and Cook, L., Discriminative stimulus properties of a σ receptor agonist in the rat: role of monoamine systems, *Neuropsychopharmacology*, 2, 201, 1989.
166. Massamiri, T. and Duckles, S.P., Sigma receptor ligands inhibit rat tail artery contractile responses by multiple mechanisms, *J. Pharmacol. Exp. Ther.*, 253, 124, 1990.
167. Yevich, J.P. et al., Synthesis and evaluation of N-substituted 1-(5-fluoro-2-pyrimidinyl)piperazine derivatives as potential anti-ischemic agents, *Bioorg. Med. Chem. Lett.*, 16(4), 1941, 1994.
168. Yevich, J.P. et al., Synthesis and biological characterization of α -(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazinebutanol and analogs as potential atypical antipsychotic agents, *J. Med. Chem.*, 35(24), 4516, 1992.
169. Patel, R.N. et al., Microbial reduction of 1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]butan-1-one, *Biotechnol. Appl. Biochem.*, 17, 139, 1993.
170. Ward, O.P. and Baev, M.V., Decarboxylases in stereoselective catalysis, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000.
171. Iding, H. et al., Application of α -keto acid decarboxylases in biotransformations, *Biochim. Biophys. Acta*, 1385, 307, 1998.
172. Iding, H. et al., Benzoylformate decarboxylase from *Pseudomonas putida* as stable catalyst for the synthesis of chiral 2-hydroxy ketones, *Chem. Eur. J.*, 6, 1483, 2000.
173. Guo, Z. et al., Asymmetric acyloin condensation catalyzed by phenylpyruvate decarboxylase, *Tetrahedron Asymmetry*, 12, 571, 2001.

174. Guo, Z. et al., Asymmetric acyloin condensation catalyzed by phenylpyruvate decarboxylase, Part 2: substrate specificity and purification of the enzyme, *Tetrahedron Asymmetry*, 12, 571, 2001.
175. Waldmann, H., Heuser, A., and Schulze S., Selective enzymic removal of protecting groups: the phenylacetamide as amino protecting group in phosphopeptide synthesis, *Tetrahedron Lett.*, 37, 8725, 1996.
176. Costello, C.A., Kreuzman, A.J., and Zmijewski, M.J., Selective deprotection of phthalyl protected amines, *Tetrahedron Lett.*, 37, 7469, 1996.
177. Bommaris, A.S. et al., L-methionine related L-amino acids by acylase cleavage of their corresponding N-acetyl-DL-derivatives, *Tetrahedron Asymmetry*, 8, 3197, 1997.
178. Ogawa, J. and Shimizu, S., Stereoselective synthesis using hydantoinases and carbamoylases, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000.
179. Ogawa, J., Miyake, H., and Shimizu S., Purification and characterization of N-carbamoyl-L-amino acid amidohydrolase with broad substrate specificity from *Alcaligenes xylosoxidans*, *Appl. Microbiol. Biotechnol.*, 43, 1039, 1995.
180. Sajiki, H., Hattori, K., and Hirota K., The formation of a novel Pd/C-ethylenediamine complex catalyst: chemoselective hydrogenation without deprotection of the O-benzyl and N-Cbz groups, *J. Org. Chem.*, 63, 7990, 1998.
181. Royer, G.P., Chow, W., and Hatton, K.S., Palladium/polyethylenimine catalysts, *J. Mol. Catal.*, 31, 1, 1985.
182. Patel, R.N. et al., Enantioselective enzymatic cleavage of N-benzyloxycarbonyl groups, *Adv. Synth. Catalysis*, 345, 830, 2003.
183. Nanduri, V.B. et al., Cloning and expression of a novel enantioselective N-carbobenzyloxy-cleaving enzyme, *Enzyme Microb. Technol.*, 34(3&4), 304, 2004.

5 Directed Evolution of Lipases and Esterases for Organic Synthesis

Aurelio Hidalgo and Uwe T. Bornscheuer

CONTENTS

5.1	Lipases and Esterases.....	159
5.1.1	Definition, Structural Features, Properties	159
5.1.2	Uses in Biocatalysis	162
5.2	Directed Evolution of Lipases and Esterases.....	162
5.2.1	Creation of Diversity.....	163
5.2.1.1	Mutagenesis Methods	163
5.2.1.2	Environmental Diversity.....	165
5.2.2	Library Screening and Selection.....	165
5.2.2.1	Screening for Hydrolytic Activity.....	166
5.2.2.2	Screening for Transesterification	167
5.2.2.3	Screening for Enantioselectivity.....	168
5.2.2.4	Screening and Selection in Solid Media.....	170
5.2.2.5	Selection as a Tool for Screening.....	170
5.3	Examples of Directed Evolution of Lipases and Esterases	172
5.3.1	Stability	172
5.3.2	Enantioselectivity	172
5.3.3	Chemoselectivity.....	174
5.4	Conclusion	175
	Acknowledgments	175
	References	175

5.1 LIPASES AND ESTERASES

5.1.1 DEFINITION, STRUCTURAL FEATURES, PROPERTIES

Lipases and esterases are, undoubtedly, enzymes with great biotechnological importance. Approximately 55% of all enzymes used in biocatalysis are hydrolases, lipases (~30%), and esterases (~8%) being the most predominant enzymes besides proteases, nitrilases, and others [1,2]. Both of these enzymes belong to the structural superfamily of α/β -hydrolases. This family groups several hydrolytic enzymes of widely differing phylogenetic origin and catalytic function. The common structural motif comprises eight beta-sheets connected by alpha-helices. These enzymes seem to diverge from a common ancestor in order to preserve the

arrangement of the catalytic residues. They all have a catalytic triad, the elements of which are borne on loops, which are the best-conserved structural features in the fold. Only the histidine in the nucleophile–histidine–acid catalytic triad is completely conserved. In the case of lipases and esterases the catalytic triad is composed of Ser-Asp-His (Ser-Glu-His for some lipases), the Ser residue being in the consensus sequence Gly-X-Ser-X-Gly. The unique topological and sequential arrangement of the triad residues produces a catalytic triad which is, in a sense, a mirror image of the serine protease catalytic triad [3].

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are the most frequently used hydrolases in organic synthesis [4,5]. They are quite widespread in nature and a considerable number of them are commercially available. In nature they catalyze the hydrolysis and transesterification of triglycerides present in fats and oils. The reaction mechanism comprises four steps detailed in Figure 5.1:

1. The substrate reacts with the active site serine, yielding a tetrahedral intermediate stabilized by the catalytic His and Asp (Glu) residues.
2. The alcohol is released and a covalent acyl–enzyme complex is formed.
3. Attack of a nucleophile (water in hydrolysis, alcohol in transesterification) forms again a tetrahedral intermediate.
4. The intermediate collapses to yield the product and regenerates the free enzyme.

Lipases are commercially used in laundry detergents, in the food industry for cheese making, for modification of fats and oils, for synthesis of sugar esters, in the cosmetics industry and personal care for the synthesis of emollient esters. A major area is the use of lipases in fine chemicals industry, as they often exhibit excellent enantio- and stereoselectivity, and, therefore, allow for the production of a range of optically active building blocks. For instance, Novozymes produces the lipases from *Candida antarctica* B (CAL-B) and *Rhizomucor miehei* (RML) for applications in organic synthesis and lipid modification, respectively [6].

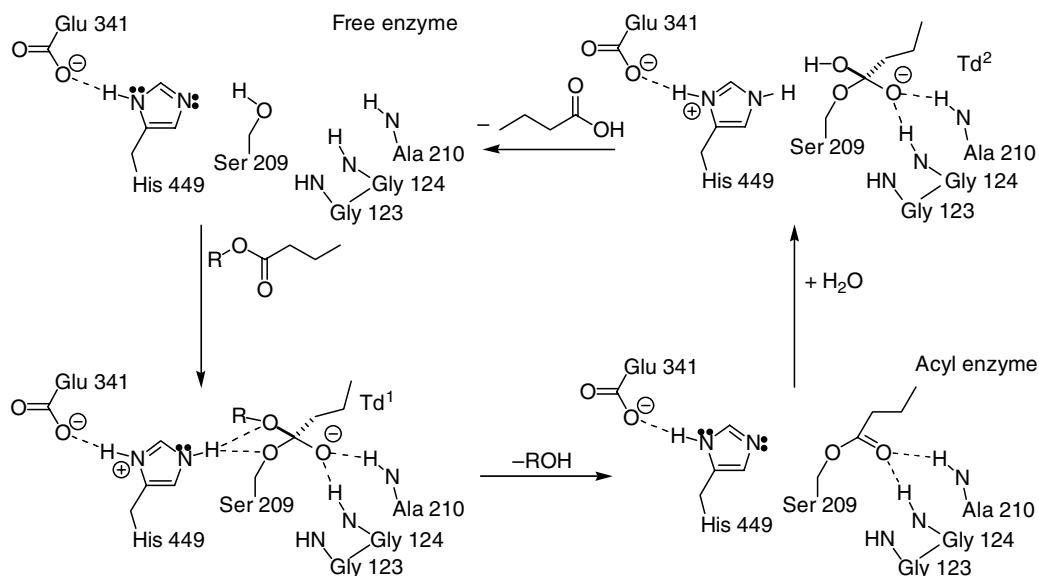


FIGURE 5.1 Hydrolysis of a butyric acid ester catalyzed by lipase or esterase. The amino acid numbering corresponds to the active site of the *Candida rugosa* lipase (CRL).

Lipases can be classified according to sequence alignment into three major groups. The mammalian lipases form one group, from which the most relevant member is the porcine pancreatic lipase (PPL). This commercial lipase preparation is an impure acetone extract from the animal source. The different proteins in the preparation exhibit different behavior in biocatalytic reactions [7] and, in fact, many of the biotransformations usually attributed to this lipase are now suspected to be originated by the contaminants in the preparation [8]. Nevertheless, the commercial preparation of PPL is currently employed in the industrial resolution of some compounds, e.g., the resolution of glycidyl butyrate developed by Ladner and Whitesides [9].

The fungal lipases can be subdivided into two groups. The first family is the *Candida rugosa* family, and it includes the lipases from *C. rugosa*, *Geotrichum candidum*, and the pancreatic cholesterol esterase (although it is not from fungal origin, the sequence similarity places it in this group). The second subgroup is the *Rhizomucor* family, which includes, amongst others, small lipases such as CAL-B, RML, and those from *Thermomyces lanuginosus* (TLL) and *Rhizopus oryzae* (ROL). For more information on the latter group, readers are referred to the review by Bornscheuer et al. [10].

Finally, the bacterial lipases can also be subdivided into two groups: the *Staphylococcus* family and the *Pseudomonas* family, which includes lipases such as *Burkholderia cepacia* lipase (BCL), *Pseudomonas fluorescens* lipase (PFL), and *Chromobacterium viscosum* lipase (CVL). Readers are also encouraged to consult the Lipase Engineering Database for further information about lipase classifications and alignments (<http://www.led.uni-stuttgart.de>).

Lipases prefer substrates containing long-chain fatty acids. Furthermore, since the natural substrate of lipases, i.e., triglycerides, are insoluble in water, lipases are able to function at the hydrophilic–hydrophobic interface. At this interface, lipases exhibit a phenomenon termed interfacial activation, which causes a remarkable increase in activity upon contact with a hydrophobic surface. This effect is due to the presence of a flexible peptide (the “flap” or “lid”) covering the active site. Lipases undergo a drastic conformational change in the presence of hydrophobic surfaces, causing the lid to displace and expose the hydrophobic active site to the interface. The catalytic process involves a series of differentiated stages: contact with the interface, conformational change, penetration in the interface, and finally the catalysis itself. The principle of interfacial activation applies not only to liquid–liquid interfaces, such as two-phase aqueous systems, but also to solid–liquid interfaces, such as in hydrophobic chromatographic supports [11,12].

Not every lipase of biotechnological interest can be produced by overexpressing the gene of interest in a heterologous host. For instance, the *Bacillus* lipases can be generally overproduced in *Escherichia coli*, but other enzymes, e.g., some from *Pseudomonas*, can hardly be overexpressed in active form in this host, and homologous expression in *Pseudomonas* sp. is preferred. The expression of fungal lipases requires careful control of codon usage and folding issues if overexpression in bacterial hosts is targeted [13]. The commercial lipases CAL-B, RML, and TLL are all expressed in *Aspergillus* sp. [14,15].

Esterases (carboxyl esterases, EC 3.1.1.1) have the same reaction mechanism as lipases, but differ from them by their substrate specificity, since they prefer short-chain fatty acids whereas lipases prefer long-chain fatty acids. Another difference lies in the “interfacial activation” phenomenon, which has been observed only for lipases. In contrast to lipases, only a few esterases have practical uses in organic synthesis, because lipases are generally more enantioselective and resistant to organic solvents, although certain examples of thermophilic esterases resistant to organic solvents have been described [16,17]. The most widely used esterase is the preparation isolated from pig liver [6] but there are several reports of kinetic resolutions of racemates catalyzed by other esterases [18–23]. Other applications of esterases include the deprotection of labile functional groups [24], the use of feruloyl esterases in pulp bleaching [25] or in vanillin production [26,27].

According to sequence similarities, Arpigny and Jaeger grouped lipases and esterases in eight families. Among them, the most remarkable groups are group I, which contains “true lipases,” mostly from Pseudomonads; group II, which comprises the GDSE-motif containing lipases and esterases; group IV, similar to the human hormone-sensitive lipase; group V, similar to epoxide hydrolases, dehalogenases, and haloperoxidase; group VI, containing some of the smallest esterases known; and group VII, which contains larger esterases [28] (see also [29]). However, such a classification based on sequence similarity cannot always explain the differences found in substrate specificity.

5.1.2 USES IN BIOCATALYSIS

Enzyme catalyzed reactions have a number of advantages that make them ideal for organic synthetic reactions. However, certain requirements should ideally be fulfilled by the biocatalysts in order to make them viable alternatives to conventional chemical processes. The biocatalyst used in such reactions should exhibit the following properties (always depending on the particular reaction):

- Chemo- and regioselectivity
- Stereoselectivity
- Desired thermostability
- Stability against organic solvents
- Easy recovery and reutilization
- Broad substrate range

Despite being overexpressed in suitable hosts, only few esterases have been used for the synthesis of optically pure compounds, because they often exhibit moderate or no enantioselectivity. Nevertheless, there are several examples described in which either wild-type or mutated esterases are used as biocatalysts to profit from their chemo-, regio-, or enantioselectivity. These have been gathered in a review about microbial carboxyl esterases by Bornscheuer [29].

Biotechnology requires enzymes that are functional and stable under a wide range of unnatural conditions [30], but enzymes do not always fulfill these requirements, and, therefore, adequate solutions have to be implemented. On one hand, reaction and medium engineering were used to change typical variables of the reaction, such as pH, temperature, type of organic cosolvent and its concentration, and immobilization support. Therefore, through a combinatorial approach, the ideal conditions can be found for every reaction studied. On the other hand, the enzyme itself can be altered to accept nonnatural substrates [31], evolved to become less prone to inactivation under harsh operating conditions [24,32–37], and even mutated to direct the immobilization toward a predetermined region of the enzyme [38]. The strategies for such alterations can either be through structural studies, ensuing modeling to decide which residues are replaced (site-directed mutagenesis), or a random approach in which the enzyme is mutated without previous knowledge of the three-dimensional structure and the variants generated are subsequently screened for the desired property, i.e., by the directed evolution approach.

5.2 DIRECTED EVOLUTION OF LIPASES AND ESTERASES

Directed evolution is a technique of enzyme alteration and selection to produce desired biocatalysts, based on the creation of random mutants that does not require prior knowledge of structure–function relationship.

A directed evolution strategy requires:

1. A mutagenesis method, as unbiased as possible (when no other constraints or requirements are preimposed)
2. A high-throughput selection method that specifically measures the property of interest, as close to the original reaction as possible

The evolution process begins with the selection of a known enzyme and of the property or activity that needs to be evolved (e.g., increased enzyme activity, improved thermal stability, higher tolerance to the presence of organic solvents, modified optimal working temperature or pH, altered specificity, and enantioselectivity). A large mutant library—usually within the range of 10^5 to 10^{10} mutants—is then prepared by mutagenesis of the entire gene, according to the techniques detailed below. After cloning and medium- or high-throughput expression, a collection of enzyme molecules is generated. This library is subjected to screening in terms of the evolved property to select the mutants exhibiting the desired characteristics. Isolated mutants might subsequently serve as improved starting points for additional rounds of mutagenesis to accumulate beneficial mutations for the best result (Figure 5.2) [39].

5.2.1 CREATION OF DIVERSITY

5.2.1.1 Mutagenesis Methods

Methods to create mutant libraries can be divided into two major categories: nonrecombining mutagenesis, in which a parent gene is subjected to random mutagenesis to yield variants with

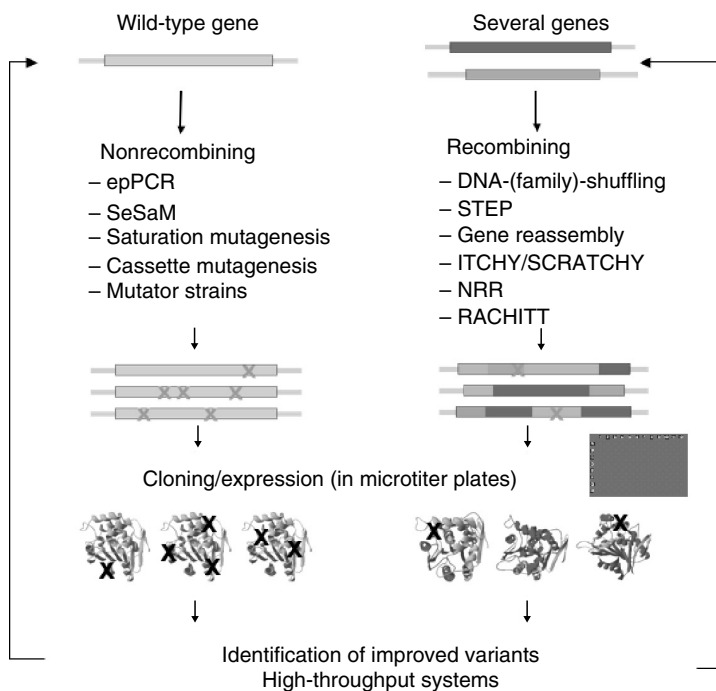


FIGURE 5.2 The process of directed evolution. For abbreviations, see text.

point mutations; and, when several parental genes (generally homologous or with a high degree of similarity) are available, they can be randomly fragmented, shuffled, and reconstructed to create a library of recombined offspring. Although an overview of some methods is presented here, readers are referred to the reviews by Neylon [40] and Kurtzman [41] for a more detailed survey.

5.2.1.1.1 *Creation of New Mutations*

Error-prone polymerase chain reaction (epPCR) is the most widespread method to generate mutations quickly and efficiently. It consists of an error-prone version of the well-known amplification reaction using DNA polymerase from thermophilic microorganisms. Under controlled conditions (addition of extra Mg^{2+} and Mn^{2+} and usage of unbalanced dNTP concentrations) a reproducible error rate is achieved. The error rate is kept low to generate adaptative mutations, since a higher error rate will lead to deleterious mutations or inactive variants. Nevertheless, a homogeneous mutational spectrum cannot be achieved using *Taq* DNA polymerase with Mn^{2+} and unbalanced nucleotides. Such a fact, known as polymerase bias, will result in a higher tendency to exchange the desoxynucleotides A and T than G and C. Improved DNA polymerases that exhibit a more homogeneous mutational spectrum are now commercially available (Stratagene) for this purpose. The bottleneck introduced by the ligation of the mutated epPCR product into a cloning or expression vector is partially solved by the “bringer” technique developed by Bichet et al., based on amplifying the whole plasmid under mutagenic conditions; but many controls are needed in order to ensure transformation of only mutated plasmids, especially of those mutated only in the target gene [42].

To overcome the positional and mutational bias connected to epPCR, a more complex approach (sequence saturation mutagenesis [SeSaM]) was developed by Wong et al. that can introduce any mutation on any position in the gene by using universal bases [43].

An easier alternative to create random mutations involves the use of mutator strains. Such strains are deficient in three of the primary DNA repair pathways and exhibit a mutation rate approximately 5000 times that of the wild-type host strain. On the one hand, with this approach, the ligation step is avoided, but on the other, there is no control on the location of the mutation, which can affect the promotor present in the plasmid, its copy number, and other features of the vector. Nevertheless, there are several references that describe successful creation of mutants using this technique [44–46].

Once a key position to enzyme function has been identified through point mutagenesis, it is a usual strategy to saturate that position, i.e., to replace the mutated amino acid for the remaining 18 proteinogenic amino acids. Furthermore, by random mutagenesis the “hot-spots” can be identified, i.e., those amino acid positions that are key to the desired property, even if they are far away from the active site, and would seem to be, at first glance, irrelevant.

However, another approach was recently developed by Reetz et al. [47] that combines randomization with site-directed mutagenesis. First, certain residues were chosen that would interact with both the acyl and alcohol in the binding pocket of *P. aeruginosa* lipase. These target amino acids were randomized in pairs, and such pairs were chosen according to structural criteria, namely, the structural motif they were part of, i.e., a loop, a sheet, or a helix. To randomize a loop, two contiguous amino acids were randomized: for a sheet, amino acids at position n and $(n + 2)$; for a 3_{10} helix, n and $(n + 3)$; and for an α -helix, n and $(n + 4)$. In this fashion, not only was the size of the library generated drastically reduced to 3000 clones per library, but the strategy also proved adequate to expand the range of substrates accepted by this enzyme.

5.2.1.1.2 *Recombination of Variants*

When beginning from already selected variants, mutagenesis methods are preferred that may combine the best features of both parental donors. These are also known as “sexual”

mutagenesis methods. The first example was developed by Stemmer [48] and termed “DNA shuffling”. It consists of a DNase-dependent degradation and subsequent recombination of the fragments without primers (self-priming PCR) followed by a final PCR with primers. An alternative to avoid bias introduced by DNase I digestion [49] is the staggered extension process (StEP) developed by Zhao et al. [50]. It consists of the amplification of short fragments of the parental genes, so that in subsequent cycles, the resulting short fragments can anneal on any other parental genes. A further improvement on the classic shuffling approach was developed by Coco et al., who devised an alternative DNA-shuffling method, random chimeragenesis on transient templates (RACHITT), based on the ordering, trimming, and joining of randomly cleaved single-stranded parental gene fragments annealed onto a transient full-length single-stranded template [51]. This method exhibited higher recombination frequencies and 100% chimerical products. Two alternative methods that focus on increasing or controlling the recombination frequency and the fraction of chimerical products over the unshuffled parental strains are the “combinatorial libraries enhanced by recombination in yeast” (CLERY) [52], which combines *in vitro* DNA shuffling with *in vivo* recombination in yeast and the degenerate oligonucleotide gene shuffling (DOGS) [53].

However, similarity of sequence exhibited by the parental genes was a necessary constraint to apply recombination techniques; several methodologies have been introduced to recombine several parental sequences without regard to homology but with control over the demarcation points, such as the Gene Reassembly method [54], the nonhomologous random recombination (NRR) [55], the incremental truncation for the creation of hybrid enzymes (ITCHY), and its variation, the thio-ITCHY [56].

As a general trend, several mutagenesis methods can be combined in order to cover the distance in the sequence space that separates the starting point (often the wild-type enzyme, but not necessarily) from the optimal variant for a given property (activity, thermostability, enantioselectivity, etc.). Thus, point mutation methods can be used in conjunction with recombination methods in order to obtain the best variant possible, for instance, by shuffling mutants containing point mutations in different regions of the gene.

5.2.1.2 Environmental Diversity

An alternative approach to create enzyme diversity is to screen libraries of environmental DNA (eDNA), also termed the “metagenomic approach” [57]. It is generally assumed that only 0.001 to 1% of all microorganisms are culturable, depending on their origin [58]. Therefore, rather than trying to isolate and culture microorganisms from diverse environments that may exhibit the desired enzyme activities, only their DNA is isolated, genomic libraries are created, and subsequently screened. All these procedures are carried out in high-throughput format [58–60]. Lipases and esterases have been isolated from environmental samples following this protocol and screened for hydrolytic activity against triolein or tributyrin [61] or directly amplified analyzing conserved regions using carefully designing primers [62]. A powerful example is the discovery of 200 nitrilases (approximately ten times more than the reported number of nitrilases up to the time of publication) from different biotopes around the globe by Diversa Corp. using high-throughput cloning and protein expression coupled with *in vivo* selection [63].

5.2.2 LIBRARY SCREENING AND SELECTION

Following creation of the mutants, these are most often cultured in solid media or in microtiter plates. The microorganisms are then incubated until enough biomass is obtained to allow for accurate and sensitive detection of enzyme activity.

A screening assay is based on the development of a detectable signal to identify a mutant by a catalytic activity that stands out from that of the background of clones in the library. This process may be directed either toward the identification and isolation of very few highly active mutants from an essentially inactive background population, or toward the quantitative measurement of the activity exhibited by each cell or colony for the identification of the most active mutants out of a moderately active background population [39]. Thus, screening methods must be sensitive to small functional changes that arise from single amino acid substitutions, and also reliable enough to distinguish one single variant among thousands [64].

Ideally, the screening assay should be based on a signal generated by the interaction between a chromogenic or fluorogenic substrate and the enzyme of interest, although indirect measurements, e.g., through coupled enzymatic reactions, are also possible [39].

Last but not least, screening methods should resemble the reaction of interest as closely as possible, to avoid the risk of selecting an enzyme that is very active against the screening (surrogate) substrate but not so active against the target substrate. This is often stated by the well-known axiom of directed evolution: *you only get what you screen for*.

In the following sections, some screening assays and selection techniques for lipases and esterases are reviewed. The assays for enantioselectivity screening in principle are special cases of methods given in [Section 5.2.2.1](#) and [Section 5.2.2.2](#). However, as the alteration of enantioselectivity is one of the most challenging targets in directed evolution, methods to determine enantioselectivity are summarized separately in [Section 5.2.2.3](#).

5.2.2.1 Screening for Hydrolytic Activity

Hydrolytic activity of esters can be determined using a wide variety of substrates, some natural and some surrogate, i.e., nonnatural substrates designed to provide an intense, detectable signal when they are converted by the enzyme. Nevertheless, not all activity assays are susceptible to being implemented in the high-throughput format required for the screening of the vast libraries created by the mutagenesis protocols used in directed evolution. An example is the simple pH-stat assay using tributyrin or triolein emulsions as substrates. Nevertheless, lipolytic activity can still be screened in a high-throughput format, on-plate, with triolein or tributyrin-agar through halo formation.

Colorimetric and fluorometric assays are undoubtedly the most widespread assays for hydrolytic activities. They involve the cleavage of an ester to yield a chromophore/fluorophore that is detected. The most commonly used chromophores/fluorophores are *p*-nitrophenol, fluorescein, resorufin, and coumarin. Some important disadvantages of hydrolytic activity assays of lipases and esterases are the rather poor solubility in aqueous media and the risk of strong autohydrolysis at extreme pH or elevated temperature using chromogenic or fluorogenic substrates. To circumvent this problem, two strategies have been described in the literature. In the first, the “traditional” esters of *p*-nitrophenol or coumarin are replaced by the corresponding acyloxymethylethers, or diacylglycerol analogs ([Figure 5.3](#)). This makes the substrate much more stable, as the ester susceptible to enzymatic cleavage is separated from the chromophore (or fluorophore), avoiding autohydrolysis since the alcohol moiety is now a worse leaving group than the coumarin or the *p*-nitrophenoxide ion. Depending on its particular structure, the cleaved alcohol is then directly decarboxylated or first oxidized with periodate and then subjected to BSA-catalyzed β -elimination in order to release the chromophore/fluorophore. Alternatively, the diols can be separated and quantified by high-performance liquid chromatography (HPLC), without elimination [65–67]. This methodology is also applicable to the screening and characterization of enantioselective enzymes [68]. Disadvantages are the need for synthesis of the specifically designed substrates and that

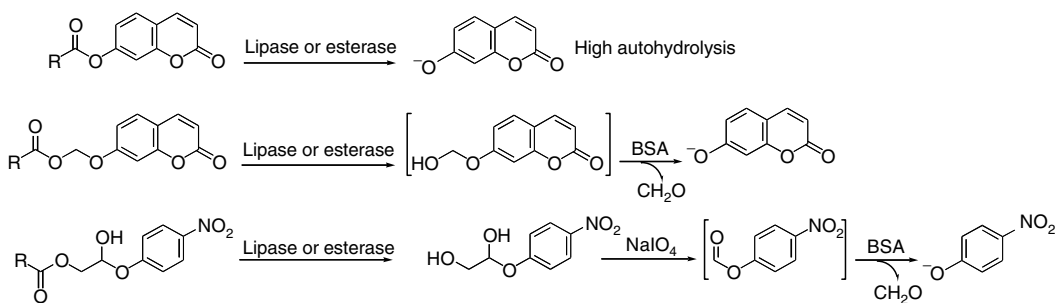


FIGURE 5.3 Fluorogenic and chromogenic substrates for lipases and esterases. The separation of the enzymatic and chromogenic reactions enables the reduction of background signal due to autohydrolysis.

only end-point measurements are possible rather than quantification of enzyme kinetics. The second strategy uses the method of back-titration with adrenaline of the sodium periodate consumed in the oxidation of the diol generated by enzymatic cleavage [69,70]. Alternatively, a high-throughput assay in solid phase was recently developed by Babiak and Reymond using esters of coumarin [71]. All these methodologies practically suppress any background reactivity of the substrates in the absence of enzyme.

A remarkably sensitive assay was recently developed by Moore et al. based on surface-enhanced resonance Raman scattering (SERRS). Although specific instrumentation for the Raman measurements is needed, the assay works in the picoliter and femtoliter range (similar to *in vivo* enzyme concentration). The principle is based on the use of a benzotriazole dye, which is masked and joined by a linker to the substrate. Lipase-catalyzed turnover of the substrate releases the dye, which complexes to dispersed silver nanoparticles and thus generates an SERRS response proportional to the enzymatic reaction. Fourteen different lipases were tested and the enantioselectivity values determined for the hydrolysis of the model compound 3-phenylbutyric acid, invariable with the dye group used. Thus, despite the use of “surrogate” substrates, not only activity but also enantioselectivity could be quantified at extremely low concentrations [72].

Very recently, an ultra-high-throughput screening method was reported by Schuster et al., based on the coexpression of pHluorin (a pH-sensitive modified green fluorescent protein) and an esterase. As the recombinant esterase catalyzes the hydrolysis of an ester, an acid is released that causes changes in the emission spectrum of pHluorin, which can be detected. However, not all the esters tested as substrate provided a significant fluorescent signal (compared to the control), and, therefore, this technique is limited to substrates not hydrolyzed by the host cell, and which can enter the cell either alone or with the help of a cosolvent. The assay in microtiter plate format was successfully implemented to a flow cytometer, in order to separate those cells expressing esterase activity from the control cells expressing an inactive esterase [73].

5.2.2.2 Screening for Transesterification

Transesterification is usually measured by gas chromatography (GC) or HPLC, and, therefore, is not susceptible to scaling up to high-throughput screening, although some examples in medium-throughput screening have been reported for other reactions [74].

A fluorometric method to determine transesterification by lipases and esterases in organic solvents was described by Konarzycka-Bessler and Bornscheuer [75]. Using vinyl acetate, the transesterification reaction is made irreversible as the vinyl alcohol undergoes keto-enol tautomerization to acetaldehyde, which reacts with 7-hydrazino-4-nitrobenzafurazane (NBDH) to yield a fluorescent hydrazone (Figure 5.4).

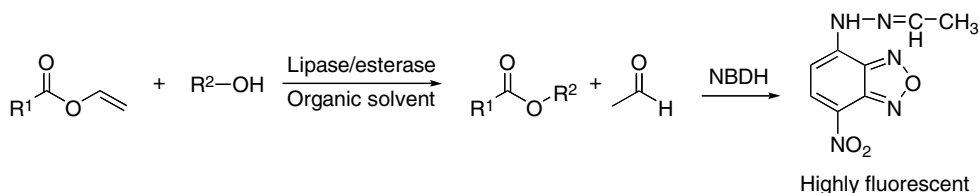


FIGURE 5.4 High-throughput assay for the determination of transesterification activity of lipases and esterases.

5.2.2.3 Screening for Enantioselectivity

Although it is well known that each enantiomer of a chiral compound may exhibit different biological activities, a number of commodity products and fine chemicals, such as drugs, agrochemicals, flavors, fragrances, and pheromones, are still used in the form of racemic mixtures. Therefore, the general trend is to replace these mixtures with single enantiomers that can be obtained directly, either by an asymmetric synthesis or by the resolution of racemates. In addition, for newly designed drugs, it may be necessary to obtain both enantiomers for pharmacological and toxicological studies [76]. Thus, enantioselectivity is often a target property for directed evolution of enzymes involved in organic synthesis of pharmaceutical compounds or synthesis intermediates. One of the main disadvantages of screening for enantioselectivity is that it depends on the reaction catalyzed by the enzymes, and not all reactions are susceptible of being implemented in a high-throughput screening setup.

In 1997, Janes et al. described the first high-throughput screening method for enantioselectivity based on the separate hydrolysis of the enantiomers of *p*-nitrophenyl esters of a chiral acid. The “Quick *E*” method determined the ratio of initial rates for each enantiomer corrected for competitive binding using simultaneously a nonchiral resorufin ester (Figure 5.5) [77]. The Quick *E* method has been applied successfully in order to test the substrate selectivity of a series of hydrolases [46,78,79].

A variation of the Quick *E* method for the determination of apparent enantioselectivity (E_{app} , based on the determination of rates using both enantiomers separately; E_{true} -values are based on the kinetic resolution of racemates, and, therefore, include competition of both enantiomers for the active site) was developed shortly afterwards using a pH indicator along with the enantiopure ester of interest. Thus, not only the need to use an unnatural chromogenic substrate but also the bias introduced by the presence of a bulky chromophore/fluorophore moiety [80] was eliminated. This principle has also been implemented on the solid phase by Copeland and Miller [81], using a fluorescent pH indicator attached to a solid

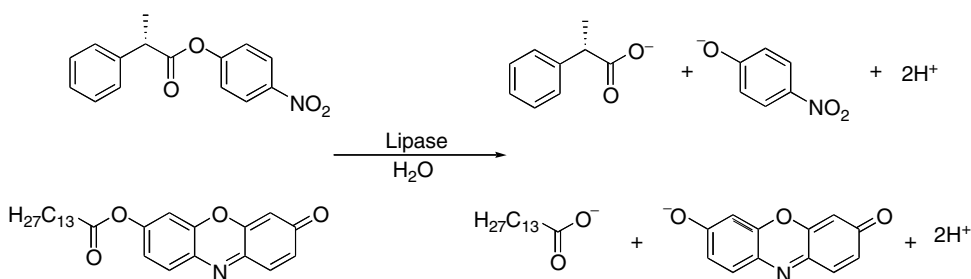


FIGURE 5.5 The “Quick *E*” method.

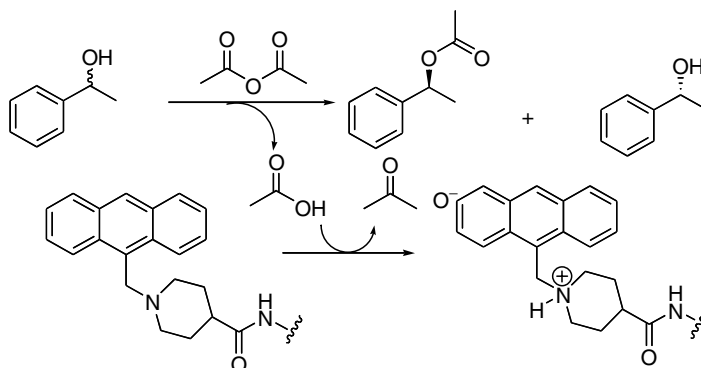


FIGURE 5.6 Screening for the enantioselective acylation of α -phenethyl alcohol with acetic anhydride, using a fluorescent indicator on solid support.

support. This assay was used to screen a catalytic peptide for the enantioselective acylation of secondary alcohols, such as α -phenethyl alcohol with acetic anhydride (Figure 5.6).

An alternative method is the commercially available “acetic acid” test (R-Biopharm GmbH, Darmstadt, Germany), which couples the hydrolysis of enantiopure chiral acetates with an acetate-dependent enzymatic cascade, leading to the formation of NADH (Figure 5.7) [82].

However, the acetic acid method can only be applied to chiral alcohols. If the chiral center is in the acyl group of an ester, alternative methods have to be used. This could be the use of *p*-nitrophenyl or resorufin esters of a chiral acid [45,83]. Nevertheless, there is a chance of selecting mutants that are optimal for the hydrolysis of these chromogenic/fluorogenic esters and not the real substrates.

All methods mentioned above make use of pure enantiomeric compounds, which are not always available. In order to use the racemate as substrate, a separation technique is needed prior to quantification of each enantiomer. This separation can be according to chirality or, in the case of isotopically labeled substrates, according to molecular mass. HPLC and GC have been adapted to high-throughput screening in order to be used [74]. With this setup, about 700 measurements were carried out per day to screen a mutant library from *P. aeruginosa* lipase toward the enantioselective esterification of 2-phenylpropanol.

Mass spectrometry (MS) is used with one isotopically labeled compound in an enantiomer pair in kinetic resolutions [84] or as shown in Figure 5.8, in the biotransformation of a *meso* compound.

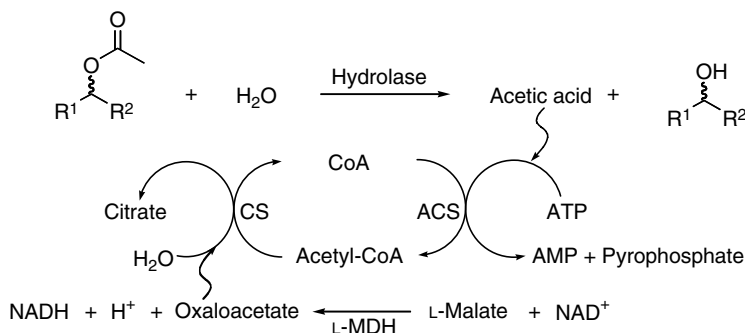


FIGURE 5.7 The “acetic acid” test.

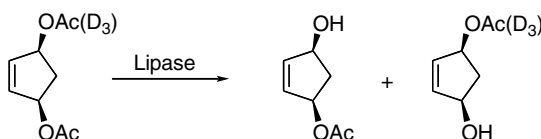


FIGURE 5.8 Kinetic resolution of an isotopically labeled *meso* compound, amenable of monitorization by mass spectrometry (MS).

Capillary electrophoresis using cyclodextrins in the electrolyte has also been adapted to process as many as 96 samples in parallel, allowing the determination of 7000 samples of derivatized chiral amines per day [85].

An elegant approach involves the use of an ELISA assay in microtiter plate format using monoclonal antibodies raised against the (*S*)-enantiomer of mandelic acid, with less than 1% cross-reactivity to the (*R*)-enantiomer [86]. Although there have not been any examples reported for lipase screening, the technique seems promising and cost-effective.

5.2.2.4 Screening and Selection in Solid Media

High-throughput screening can be performed directly on the colonies expressing enzyme variants in a solid culture (agar plate) or in membranes when cell lysis is required. When screening in solid and not liquid medium, the formation of insoluble, colored products is preferred, e.g., X-gal or α -naphthyl acetate and Fast Blue overlay agar assay for esterase activity detection [87]. An example of the above-mentioned strategy is the selection of mutants of *P. fluorescens* esterase I (PFEI) created with the mutator strain XL-1 Red for the resolution of an intermediate in the synthesis of epothilones. In this case the hydrolysis of the adequate ester was evidenced by the presence of a pH indicator in the solid medium coupled with growth enhancement of the colonies producing an esterase with the adequate selectivity [44].

5.2.2.5 Selection as a Tool for Screening

Mutagenesis techniques create libraries often ranging between 10^5 and 10^{10} individuals. In order to screen a significant amount of sequence space, the size of the library can be reduced by discarding inactive or uninteresting mutants. Such selection can be carried out *in vitro* or *in vivo*.

Phage display is one of the most common techniques for *in vitro* selection of the best mutants in a large library (Figure 5.9). It involves cloning of the gene of interest (in this case every individual in a library of mutants) in fusion with a gene encoding a coat protein of the virion. When the phage is assembled, the foreign protein is displayed on its surface. Therefore, a physical linkage between gene and expression product is achieved by means of a phage particle. The phages are then captured by affinity interaction of the displayed enzyme with an immobilized ligand. The nature of this binding depends on the enzyme, e.g., the tag can be a substrate, a suicide-substrate coupled to biotin (which is subsequently captured on Streptavidin beads), or an immobilized transition state analog. The selected phages are eluted, replicated, and amplified by simple infection [88]. Danielsen et al. illustrated the selection of Lipolase displaying phages with a biotinylated phosphorylating inhibitor that enables to enrich the library by 180-fold in a single round [89].

Protein libraries can be displayed not only on the surface of bacteriophages but also on bacteria and yeast. Bacterial display presents certain advantages over the much more widespread phage display. First, only one host is needed to propagate the library, compared with

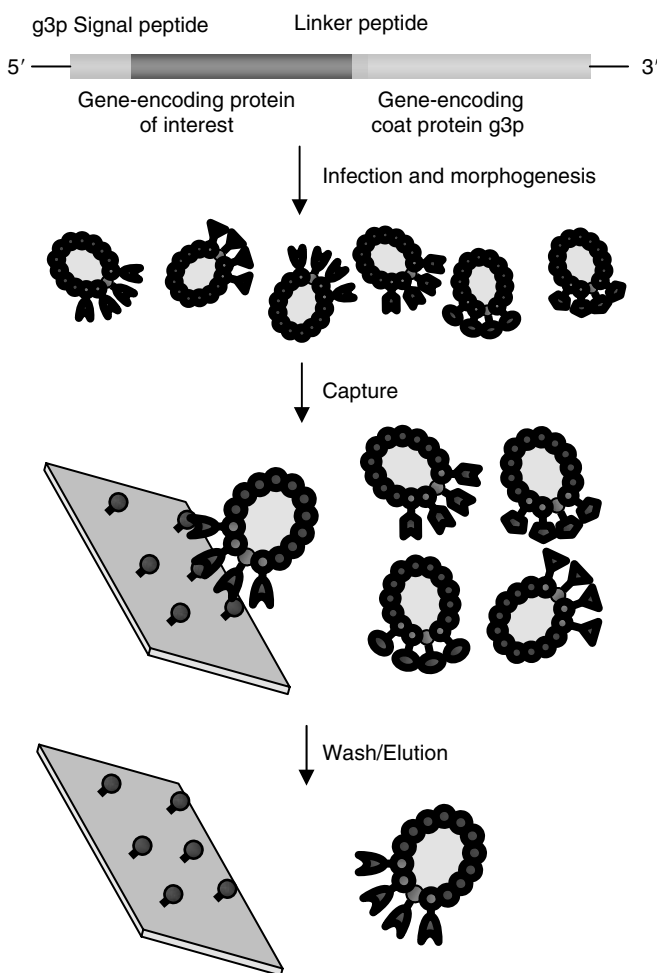


FIGURE 5.9 The procedure of phage display.

two—the bacteriophage and bacterium—in phage display. Second, the selected variants can be directly amplified without further transfer of the genetic material to another host. Third, the risk of affinity artifacts due to avidity effects might be less pronounced. Fourth, direct screening using fluorescence-activated cell sorter (FACS) is possible [90]. Bacterial display of an esterase from *B. gladioli* was achieved by Schultheiss et al. using an artificial gene composed of the esterase gene and the essential autotransporter domains in *E. coli*. The esterase activity was successfully directed to the outer-membrane fraction as confirmed by different techniques [91].

In vivo selection can be postulated when the target activity is essential for viability, and growth (e.g., overcoming increasing concentrations of antibiotics or providing an essential nutrient) selection might be applicable [92].

The principle is illustrated by Reetz and Rüggeberg with an example in which survival is coupled to the hydrolysis of a certain enantiomer that releases a growth-inhibiting compound [93]. Thus, microorganisms expressing the lipase variant with the adequate enantioselectivity will not cleave this compound, and, therefore, survive, promoting an effective enrichment of the culture in the enzyme variant with the desired enantioselectivity.

5.3 EXAMPLES OF DIRECTED EVOLUTION OF LIPASES AND ESTERASES

In this section, we will detail some examples in which lipases and esterases have been evolved to obtain more stable variants under certain denaturing conditions (presence of organic solvents, high temperature), variants more enantioselective in a certain reaction, and enzyme variants more active than the wild type toward a new substrate.

5.3.1 STABILITY

Already during 1991–1993 the pioneering work of Chen and Arnold applied basic directed evolution principles to evolve the protease subtilisin toward activity in the presence of an organic solvent [32,33], but it was not until 1996 that the first esterase was evolved. The *p*-nitrobenzyl esterase from *Bacillus subtilis* (pNBE) was altered by several rounds of random mutation and DNA shuffling to catalyze the deprotection of the antibiotic nucleus loracarbef in 15% dimethylformamide (DMF) with 150 times higher activity compared to the wild type (Figure 5.10) [24].

In a separate study by the same group, pNBE was evolved by means of eight rounds of epPCR and recombination to yield a variant with a melting temperature 17°C higher than the wild-type enzyme, and increased hydrolytic activity toward *p*-nitrophenyl acetate (pNPA) [34,37].

C. antarctica lipase B (CAL-B) is one of the most used biocatalysts in organic chemistry. Nevertheless, it was not possible to obtain more thermostable variants by rational mutagenesis. When the directed evolution approach was used, after two rounds of mutation by epPCR, variants that were 20-fold more stable at 70°C than the wild type were found. Positions 221 and 281 were found to be critical to prevent irreversible inactivation and protein aggregation in these enhanced variants, which also proved to be more active against *p*-nitrophenyl butyrate and 6,8-difluoro-4-methylumbelliferyl octanoate [35].

5.3.2 ENANTIOSELECTIVITY

One of the main applications of lipases and esterases in organic synthesis is the resolution of racemates since the three-dimensional structures of the active site and binding sites of enzymes provide a natural scaffold for enantiorecognition.

Shortly after the report of the directed evolution of pNBE, the first example of directed evolution of a lipase (from *P. aeruginosa*) was reported by Reetz et al. for the kinetic resolution of the racemic ester detailed in Figure 5.11 [94].

The initial enantioselectivity factor, *E*, for this transformation was 1.1 (in favor of the (*S*)-acid), and after four rounds an *E* of 11.3 was obtained. Further mutants were created by combining mutations on the positions identified to be critical in the generation of the best variants for every round, which led to the identification of a more enantioselective variant (*E* = 21) [95]. For the same reaction, a DNA shuffling approach proved effective, yielding a

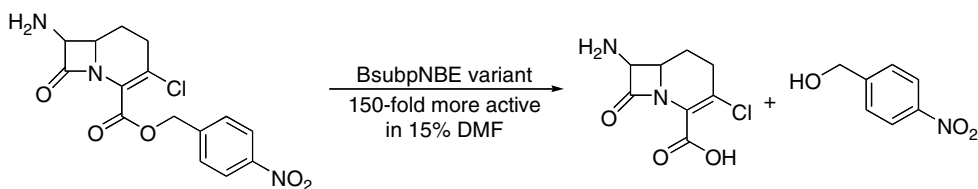


FIGURE 5.10 Deprotection of loracarbef by BsubpNBE and its variants in 15% dimethylformamide (DMF).

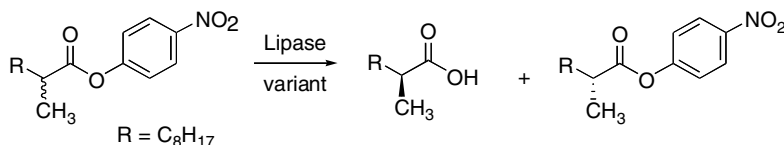


FIGURE 5.11 Resolution of a racemic ester by an evolved lipase from *Pseudomonas aeruginosa*.

variant that exhibited $E = 32$. Furthermore, a modified version of Stemmer's combinatorial multiple-cassette mutagenesis was applied to two of the obtained mutants and a mutagenic oligocassette that allowed simultaneous randomization at previously determined "hot spots." This resulted in the most enantioselective variant, displaying a selectivity factor of $E = 51$. In addition, variants with good (*R*)-selectivity ($E = 30$) were also identified [96].

A simple and elegant example is the generation of enantioselective mutants for the hydrolytic kinetic resolution illustrated in Figure 5.12 using the mutator strain *E. coli* XL-1 Red [44].

An attempt to rationalize the enantiopreference toward primary and secondary alcohols was developed by Kazlauskas et al. and is commonly known as the "Kazlauskas Rule" [97]. This rule is mainly based on the size of substituents attached to the stereogenic center. High E -values are achieved only when a small (i.e., methyl-) and a large (i.e., phenyl-) substituent are present. Thus, secondary alcohols with residues having only small differences in size are "difficult to resolve."

Attempts to resolve racemic mixtures of esters of secondary alcohols with mutants of the *P. fluorescens* esterase I have been reported. Henke and Bornscheuer evolved this enzyme using the mutator strain *E. coli* XL-1 Red, to develop enantiopreference in the hydrolysis of methyl 3-phenylbutyrate [45]. The same enzyme was evolved by a single round of epPCR to obtain a variant containing three point mutations that could be used in the kinetic resolution of the acetic acid 1-methyl-prop-2-ynyl ester. Subsequently, the role of each mutation on the enantioselectivity, the reaction rate, and the solubility of the mutant was studied [98].

Regarding esters of tertiary alcohols, a specific amino acid motif (GGGX) is needed in the oxyanion pocket of lipases and esterases in order for them to be accepted as substrates. Nevertheless, their natural enantioselectivity is still low but can be optimized through rational protein design, as exemplified by Henke et al. to increase the enantioselectivity of an esterase from *B. subtilis* from $E = 3$ to an E -value of 19 [99].

CAL-B was also engineered by shuffling its gene with those of lipases from *Hyphozyma* sp. CBS 648.91 and *Cryptococcus tsukubaensis* ATCC 24555 in order to create a lipase B variant with increased activity against the hydrolysis of diethyl 3-(3',4'-dichlorophenyl)glutamate, which yields a chiral synthon for the preparation of an NK1/NK2 dual antagonist (Figure 5.13).

The work of Koga et al. illustrates that directed evolution could also be carried out without the need for an *in vivo* expression system. Using a novel technique for the construction and screening of a protein library by single-molecule DNA amplification by PCR

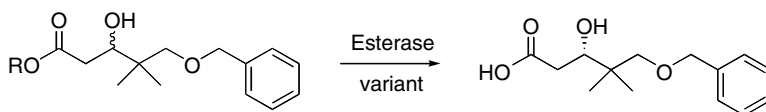


FIGURE 5.12 Kinetic resolution of a precursor ester for epothilone synthesis by *Pseudomonas fluorescens* esterase I evolved using a mutator strain.

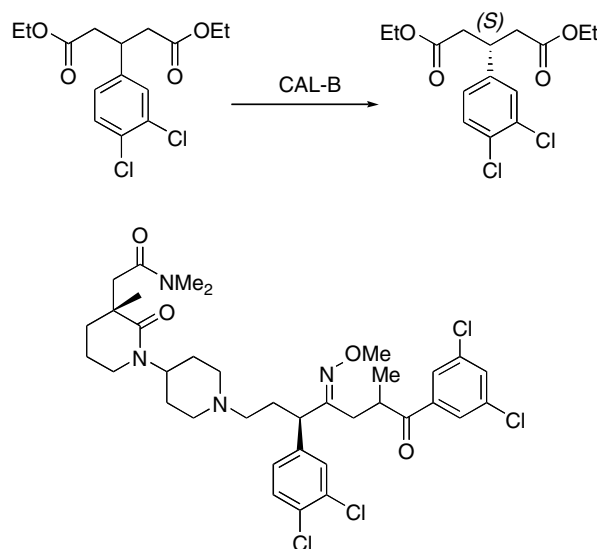


FIGURE 5.13 Hydrolysis of diethyl 3-(3',4'-dichlorophenyl)glutarate by a lipase CAL-B variant with increased activity, to obtain a chiral synthon for the preparation of an NK1/NK2 dual antagonist.

followed by *in vitro* coupled transcription/translation system termed single-molecule-PCR-linked *in vitro* expression (SIMPLEX), the enantioselectivity of *B. cepacia* KWI-56 lipase was evolved toward (*R*)-enantioselectivity in the hydrolysis of 3-phenylbutyric acid *p*-nitrophenyl ester. The library was generated saturating four positions (L17, F119, L167, and L266), and then diluted until only five molecules of DNA were present per well in the microtiter plate. These molecules were amplified using a single-molecule PCR product and expressed *in vitro*, since each gene fragment already carries a T7 promoter, a ribosome-binding site, and a T7 terminator. The DNA corresponding to active wells showing the desired enantioselectivity was once again diluted to give one molecule per well, reamplified, and rechecked. The best mutant exhibited a selectivity factor of $E = 38$ toward the (*R*)-enantiomer, whereas the wild type exhibited $E = 33$ for the opposite enantiomer [100].

5.3.3 CHEMOSELECTIVITY

Directed evolution can be used to alter the regiospecificity of a reaction, having the enzyme accept other functional groups different than the one which is its natural substrate. Some enzymes already catalyze reactions on alternative functional groups, but at a very slow rate, compared to their main catalytic function. Such behavior, termed “catalytic promiscuity” could be the basis for a directed evolution strategy aimed at altering the chemoselectivity of an enzyme. For further information on this concept, readers are referred to the works of Bornscheuer and Kazlauskas [101] and Aharoni et al. [102].

Phospholipase A₁ activity—already present in many lipases as promiscuous activity—was substantially enhanced in the *Staphylococcus aureus* lipase (SAL) by sequential rounds of epPCR. After four rounds, two products were obtained, displaying a 5.9- and 6.9-fold increase in phospholipase/lipase activity ratio. A final round of DNA shuffling with these two products and wild-type SAL was performed to combine beneficial mutations and to eliminate neutral or deleterious mutations. This procedure yielded a variant containing six amino acid mutations displaying a 11.6-fold increase in absolute phospholipase activity and a 11.5-fold increase in phospholipase/lipase ratio compared to the starting point [103]. In a

similar way, a single round of epPCR yielded a 17-fold increase in the phospholipase/lipase ratio of the thermoalkalophilic lipase of *B. thermocatenulatus* [104].

Recently, Fujii et al. reported the enhancement of amidase activity of *P. aeruginosa* lipase after one single round of random mutagenesis. Mutant libraries were screened for hydrolytic activity against oleyl-naphthylamide compared to the hydrolysis rate of the corresponding ester. Three mutational sites were identified to enhance amidase activity, and the double mutant F207S/A213D was found to have the highest amidase activity, twofold that of the wild type. These mutations were located near the calcium binding site, far from the active site [31].

The chain length selectivity of lipases can be altered by site-directed mutagenesis, such as exemplified by Joerger and Haas for the *R. oryzae* (formerly *R. delemar*) lipase (RDL) [105], and also by directed evolution [89]. While trying to isolate new enzyme variants of the extracellular lipase from *T. lanuginosa* with enhanced activity in the presence of detergent, Danielsen et al. randomized nine amino acids in two regions flanking the flexible α -helical lid. A S83T mutation was found in six of the seven most active variants, which in the homologous RDL had been proven to alter the chain length preference.

5.4 CONCLUSION

Considerable progress has been made in the past few years with respect to discovery and improvement of lipases and esterases for biocatalysis. Directed evolution and the necessary methods for library creation and high-throughput screening stimulated the research for even better hydrolases. The examples covered in this chapter demonstrate that indeed many significant achievements were made leading to enzymes with often substantially improved properties—especially with respect to enantioselectivity—further extending the possibility for applying them in biocatalytic processes.

ACKNOWLEDGMENTS

We acknowledge financial support provided through the European Community's Human Potential Programme under contract HPRN-CT2002-00239.

REFERENCES

1. Faber, K., *Biotransformations in Organic Chemistry*, 5th ed., Springer, Berlin, 2004.
2. Lloyd, R.C. et al., Use of hydrolases for the synthesis of cyclic amino acids, *Tetrahedron*, 60, 714, 2004.
3. Ollis, D. et al., The alpha/beta hydrolase fold, *Protein Eng.*, 5, 197, 1992.
4. Kazlauskas, R.J. and Bornscheuer, U.T., *Biotransformation with Lipases*, in *Biotechnology Series*, vol. 8a, Kelly, D.R., Ed., Wiley-VCH, Weinheim, Germany, 1998, p. 37.
5. Schmid, R.D. and Verger, R., Lipases: interfacial enzymes with attractive applications, *Angew. Chem. Int. Ed.*, 37, 1608, 1998.
6. Bornscheuer, U.T. and Kazlauskas, R.J., *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations*, 2nd ed., Wiley-VCH, Weinheim, Germany, 2005.
7. Segura, R.L. et al., Different properties of the lipases contained in porcine pancreatic lipase extracts as enantioselective biocatalysts, *Biotechnol. Prog.*, 20, 825, 2004.
8. Segura, R.D.L. et al., Purification and identification of the enzyme responsible of most esterase activity in commercial porcine pancreatic lipase preparations, *Enzyme Microb. Technol.*, 39, 817, 2006.
9. Ladner, W.E. and Whitesides, G., Lipase-catalyzed hydrolysis as a route to esters of chiral epoxy alcohols, *J. Am. Chem. Soc.*, 106, 7250, 1984.

10. Bornscheuer, U.T. et al., Lipases from *Rhizopus* species: genetics, structures and applications, in *Protein Engineering in Industrial Biotechnology*, Alberghina L., Ed., Harwood Academic Publishers, Amsterdam, 2000.
11. Bastida, A. et al., A single step purification, immobilization, and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports, *Biotechnol. Bioeng.*, 58, 486, 1998.
12. Palomo, J.M. et al., Purification, immobilization, and stabilization of a lipase from *Bacillus thermocatenulatus* by interfacial adsorption on hydrophobic supports, *Biotechnol. Prog.*, 20, 630, 2004.
13. Jaeger, K.-E. and Eggert, T., Lipases for biotechnology, *Curr. Opin. Biotechnol.*, 13, 390, 2002.
14. Høge-Jensen, B. et al., *Rhizomucor miehei* triglyceride lipase is processed and secreted from transformed *Aspergillus oryzae*, *Lipids*, 24, 781, 1989.
15. Hoegh, I. et al., Two lipases from *Candida antarctica*—cloning and expression in *Aspergillus oryzae*, *Can. J. Botan.*, 73, S869, 1995.
16. Sobek, H. and Gorisch, H., Purification and characterization of a heat stable esterase from the thermoacidophilic archaeobacterium *Sulfolobus acidocaldarius*, *Appl. Environ. Microbiol.*, 61, 729, 1988.
17. Luthi, E., Jasmat, N.B., and Bergquist, P.L., Overproduction of an acetylxyylan esterase from the extreme thermophilic bacterium *Caldocellum saccharolyticum*, *Appl. Microbiol. Biotechnol.*, 19, 2677, 1990.
18. Nishizawa, M. et al., Stereoselective production of (+)-*trans*-chrysanthemic acid by a microbial esterase: cloning, nucleotide sequence, and overexpression of the esterase gene of *Arthrobacter globiformis* in *Escherichia coli*, *Appl. Environ. Microbiol.*, 61, 3208, 1995.
19. Quax, W.J. and Broekhuizen, C.P., Development of a new *Bacillus* carboxyl esterase for use in the resolution of chiral drugs, *Appl. Microbiol. Biotechnol.*, 41, 425, 1994.
20. Krebsfänger, N., Schierholz, K., and Bornscheuer, U.T., Enantioselectivity of a recombinant esterase from *Pseudomonas fluorescens* towards alcohols and carboxylic acids, *J. Biotechnol.*, 60, 105, 1998.
21. Krebsfänger, N. et al., Characterization and enantioselectivity of a recombinant esterase from *Pseudomonas fluorescens*, *Enzyme Microb. Technol.*, 22, 641, 1998.
22. Baumann, M., Hauer, B.H., and Bornscheuer, U.T., Rapid screening of hydrolases for the enantioselective conversion of “difficult-to-resolve” substrates, *Tetrahedron Asymmetry*, 11, 4781, 2000.
23. Hou, C.T., Screening of microbial esterases for asymmetric hydrolysis of 2-ethylhexylbutyrate, *J. Ind. Microbiol.*, 11, 73, 1993.
24. Moore, J.C. and Arnold, F.H., Directed evolution of a *para*-nitrobenzyl esterase for aqueous-organic solvents, *Nat. Biotechnol.*, 14, 458, 1996.
25. Record, E. et al., Overproduction of the *Aspergillus niger* feruloyl esterase for pulp bleaching application, *Appl. Microbiol. Biotechnol.*, 62, 349, 2003.
26. Priefert, H., Rabenhorst, J., and Steinbüchel, A., Biotechnological production of vanillin, *Appl. Microbiol. Biotechnol.*, 56, 296, 2001.
27. Cheetham, P.S.J., Gradley, M.L., and Sime, J.T., Flavour/aroma materials and their preparation, PCT Patent, WO0050622, 2000.
28. Arpigny, J.L. and Jaeger, K.-E., Bacterial lipolytic enzymes: classification and properties, *Biochem. J.*, 343, 177, 1999.
29. Bornscheuer, U.T., Microbial carboxyl esterases: classification, properties and application in biocatalysis, *FEMS Microbiol. Rev.*, 26, 73, 2002.
30. Eijssink, V.G.H. et al., Directed evolution of enzyme stability, *Biomol. Eng.*, 22, 21, 2005.
31. Fujii, R. et al., Directed evolution of *Pseudomonas aeruginosa* lipase for improved amide-hydrolyzing activity, *Prot. Eng. Des. Sel.*, 18, 93, 2005.
32. You, L. and Arnold, F.H., Directed evolution of subtilisin E in *Bacillus subtilis* to enhance total activity in aqueous dimethylformamide, *Protein Eng.*, 9, 77, 1993.
33. Chen, K. and Arnold, F.H., Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide, *Proc. Natl. Acad. Sci. USA*, 90, 5618, 1993.

34. Gershenson, A. et al., Tryptophan phosphorescence study of enzyme flexibility and unfolding in laboratory-evolved thermostable esterases, *Biochemistry*, 39, 4658, 2000.
35. Zhang, N. et al., Improving tolerance of *Candida antarctica* lipase B towards irreversible thermal inactivation through directed evolution, *Protein Eng.*, 16, 599, 2003.
36. Spiller, B. et al., A structural view of evolutionary divergence, *Proc. Natl. Acad. Sci. USA*, 96, 12305, 1999.
37. Giver, L. et al., Directed evolution of a thermostable esterase, *Proc. Natl. Acad. Sci. USA*, 95, 12809, 1998.
38. Abián, O. et al., Stabilization of penicillin G acylase from *Escherichia coli*: site-directed mutagenesis of the protein surface to increase multipoint covalent attachment, *Appl. Environ. Microbiol.*, 70, 1249, 2004.
39. Cohen, N. et al., *In vitro* enzyme evolution: the screening challenge of isolating the one in a million, *Trends Biotechnol.*, 19, 507, 2001.
40. Neylon, C., Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution, *Nucleic Acids Res.*, 32, 1448, 2004.
41. Kurtzman, A.L. et al., Advances in directed protein evolution by recursive genetic recombination: applications to therapeutic proteins, *Curr. Opin. Biotechnol.*, 12, 361, 2001.
42. Bichet, A. et al., The “Bringer” strategy: a very fast and highly efficient method for construction of mutant libraries by error-prone polymerase chain reaction of ring-closed plasmids, *Appl. Biochem. Biotechnol.*, 117, 115, 2004.
43. Wong, T.S. et al., Sequence saturation mutagenesis (SeSaM): a novel method for directed evolution, *Nucleic Acids Res.*, 32, e26, 2004.
44. Bornscheuer, U.T., Altenbuchner, J., and Meyer, H.H., Directed evolution of an esterase for the stereoselective resolution of a key intermediate in the synthesis of epothilones, *Biotechnol. Bioeng.*, 58, 554, 1998.
45. Henke, E. and Bornscheuer, U.T., Directed evolution of an esterase from *Pseudomonas fluorescens*: random mutagenesis by error-prone PCR or a mutator strain and identification of mutants showing enhanced enantioselectivity by a resorufin-based fluorescence assay, *Biol. Chem.*, 380, 1029, 1999.
46. Horsman, G.P. et al., Mutations in distant residues moderately increase the enantioselectivity of *Pseudomonas fluorescens* esterase towards methyl 3-bromo-2-methyl propanoate and ethyl 3-phenylbutyrate, *Chem. Eur. J.*, 9, 1933, 2003.
47. Reetz, M.T. et al., Expanding the range of substrate acceptance of enzymes: combinatorial active-site saturation test, *Angew. Chem. Int. Ed.*, 44, 4192, 2005.
48. Stemmer, W.P.C., Rapid evolution of a protein by *in vitro* DNA shuffling, *Nat. Biotechnol.*, 370, 389, 1994.
49. Joern, J.M., Meinhold, P., and Arnold, F.H., Analysis of shuffled gene libraries, *J. Mol. Biol.*, 316, 643, 2002.
50. Zhao, H., Molecular evolution by staggered extension process (StEP) *in vitro* recombination, *Nat. Biotechnol.*, 16, 258, 1998.
51. Coco, W.M. et al., DNA shuffling methods for generating highly recombined genes and evolved enzymes, *Nat. Biotechnol.*, 19, 354, 2001.
52. Abecassis, V., Pompon, D., and Truan, G., High efficiency family shuffling based on multi-step PCR and *in vivo* DNA recombination in yeast: statistical and functional analysis of a combinatorial library between human cytochrome P450 1A1 and 1A2, *Nucleic Acids Res.*, 28, e88, 2000.
53. Bergquist, P.L., Reeves, R.A., and Gibbs, M.D., Degenerate oligonucleotide gene shuffling (DOGS) and random drift mutagenesis (RNDM): two complementary techniques for enzyme evolution, *Biomol. Eng.*, 22, 63, 2005.
54. Short, J.M., Synthetic ligation reassembly in directed evolution, U.S. Patent, US6537776, 2003.
55. Bittker, J.A. et al., Directed evolution of protein enzymes using nonhomologous random recombination, *Proc. Natl. Acad. Sci. USA*, 101, 7011, 2004.
56. Lutz, S., Ostermeier, M., and Benkovic, S.J., Rapid generation of incremental truncation libraries for protein engineering using alpha phosphothioate nucleotides, *Nucleic Acids Res.*, 29, e16, 2001.
57. Lorenz, P. and Eck, J., Metagenomics and industrial applications, *Nature*, 3, 510, 2005.

58. Lorenz, P. et al., The impact of non-cultivated biodiversity on enzyme discovery and evolution, *Biocat. Biotransform.*, 21, 87, 2003.
59. Uchiyama, T. et al., Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes, *Nat. Biotechnol.*, 23, 88, 2005.
60. Handelsman, J., Sorting out metagenomes, *Nat. Biotechnol.*, 23, 38, 2005.
61. Henne, A. et al., Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on *Escherichia coli*, *Appl. Environ. Microbiol.*, 66, 3113, 2000.
62. Bell, P.J.L. et al., Prospecting for novel lipase genes using PCR, *Microbiology*, 148, 2283, 2002.
63. Robertson, D.E. et al., Exploring nitrilase sequence space for enantioselective catalysis, *Appl. Environ. Microbiol.*, 70, 2429, 2004.
64. Arnold, F.H. et al., How enzymes adapt: lessons from directed evolution, *Trends Biochem. Sci.*, 26, 100, 2001.
65. Leroy, E., Bensen, N., and Reymond, J.-L., A low background high-throughput screening (HTS) fluorescence assay for lipases and esterases using acyloxymethylethers of umbelliferone, *Bioorg. Med. Chem. Lett.*, 13, 2105, 2003.
66. Klein, G. and Reymond, J.-L., An enantioselective fluorimetric assay for alcohol dehydrogenases using albumin-catalyzed β -elimination of umbelliferone, *Bioorg. Med. Chem. Lett.*, 8, 1113, 1998.
67. Grognum, J. et al., Universal chromogenic substrates for lipases and esterases, *Tetrahedron Asymmetry*, 15, 2981, 2004.
68. Grognum, J. and Reymond, J.-L., Classifying enzymes from selectivity fingerprints, *Chem. Biochem.*, 5, 826, 2004.
69. Wahler, D. et al., Adrenaline profiling of lipases and esterases with 1,2-diol and carbohydrate acetates, *Tetrahedron*, 60, 703, 2004.
70. Wahler, D. and Reymond, J.-L., The adrenaline test for enzymes, *Angew. Chem. Int. Ed.*, 41, 1229, 2002.
71. Babiak, P. and Reymond, J.L., A high-throughput, low-volume enzyme assay on solid support, *Anal. Chem.*, 77, 373, 2005.
72. Moore, B.D. et al., Rapid and ultra-sensitive determination of enzyme activities using surface-enhanced resonance Raman scattering, *Nat. Biotechnol.*, 22, 1133, 2004.
73. Schuster, S. et al., pHluorin-based *in vivo* assay for hydrolase screening, *Anal. Chem.*, 77, 2727, 2005.
74. Reetz, M.T. et al., A GC-based method for high-throughput screening of enantioselective catalysts, *Catal. Today*, 67, 389, 2001.
75. Konarczycka-Bessler, M. and Bornscheuer, U.T., A high-throughput-screening method for determining the synthetic activity of hydrolases, *Angew. Chem. Int. Ed.*, 42, 1418, 2003.
76. Murer, P. et al., Combinatorial "library on bead" approach to polymeric materials with vastly enhanced chiral recognition, *Chem. Commun.*, 2559, 1998.
77. Janes, L.E., Kazlauskas, R.J., and Quick, E., A fast spectroscopic method to measure the enantioselectivity of hydrolases, *J. Org. Chem.*, 62, 4560, 1997.
78. Liu, A.M.F. et al., Mapping the substrate selectivity of new hydrolases using colorimetric screening: lipases from *Bacillus thermocatenulatus* and *Phiostruma piliferum*, esterases from *Pseudomonas fluorescens* and *Streptomyces diastatochromogenes*, *Tetrahedron Asymmetry*, 12, 545, 2001.
79. Somers, N.A. and Kazlauskas, R.J., Mapping the substrate selectivity and enantioselectivity of esterases from thermophiles, *Tetrahedron Asymmetry*, 15, 2991, 2004.
80. Janes, L.E., Löwendahl, C., and Kazlauskas, R.J., Quantitative screening of hydrolase libraries using pH indicators: identifying active and enantioselective hydrolases, *Chem. Eur. J.*, 4, 2324, 1998.
81. Copeland, G.T. and Miller, S.J., Selection of enantioselective acyl transfer catalysts from a pooled peptide library through a fluorescence-based activity assay: an approach to kinetic resolution of secondary alcohols of broad structural scope, *J. Am. Chem. Soc.*, 123, 6496, 2001.
82. Baumann, M., Stürmer, R., and Bornscheuer, U.T., A high-throughput-screening method for the identification of active and enantioselective hydrolases, *Angew. Chem. Int. Ed.*, 40, 4201, 2001.
83. Reetz, M.T. et al., Creation of enantioselective biocatalysts for organic chemistry by *in vitro* evolution, *Angew. Chem. Int. Ed.*, 36, 2830, 1997.

84. Reetz, M.T. et al., A method for high-throughput screening of enantioselective catalysts, *Angew. Chem. Int. Ed.*, 38, 1758, 1999.
85. Reetz, M.T. et al., Super-high-throughput screening of enantioselective catalysts by using capillary array electrophoresis, *Angew. Chem. Int. Ed.*, 39, 3891, 2000.
86. Taran, F. et al., High-throughput screening of enantioselective catalysts by immunoassay, *Angew. Chem. Int. Ed.*, 41, 124, 2002.
87. Higerd, T.B. and Spizizen, J., Isolation of two acetyl esterases from extracts of *Bacillus subtilis*, *J. Bacteriol.*, 114, 1184, 1973.
88. Soumillon, P., Selection of phage-displayed enzymes, in *Evolutionary Methods in Biotechnology: Clever Tricks for Directed Evolution*, Brakmann, S. and Schwienhorst, A., Eds., Wiley-VCH, Weinheim, Germany, 2004.
89. Danielsen, S. et al., *In vitro* selection of enzymatically active lipase variants from phage libraries using a mechanism-based inhibitor, *Gene*, 272, 267, 2001.
90. Samuelson, P. et al., Display of proteins on bacteria, *J. Biotechnol.*, 96, 129, 2002.
91. Schultheiss, E. et al., Functional esterase surface display by the autotransporter pathway in *Escherichia coli*, *J. Mol. Catal. B: Enzym.*, 18, 89, 2002.
92. Lorenz, P. and Eck, J., Screening for novel industrial biocatalysts, *Eng. Life Sci.*, 4, 501, 2004.
93. Reetz, M.T. and Rüggeberg, C.J., A screening system for enantioselective enzymes based on differential cell growth, *Chem. Commun.*, 7, 1428, 2002.
94. Reetz, M.T., An overview of high-throughput screening systems for enantioselective enzymatic transformations, in *Directed Enzyme Evolution: Screening and Selection Methods*, Arnold, F.H. and Georgiou, G., Eds., Humana Press, Totowa, NJ, p. 259.
95. Liebeton, K. et al., Directed evolution of an enantioselective lipase, *Chem. Biol.*, 7, 709, 2000.
96. Reetz, M.T. et al., Directed evolution of an enantioselective enzyme through combinatorial multiple-cassette mutagenesis, *Angew. Chem. Int. Ed.*, 40, 3589, 2001.
97. Kazlauskas, R.J. et al., A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*, *J. Org. Chem.*, 56, 2656, 1991.
98. Schmidt, M. et al., Directed evolution of an esterase from *Pseudomonas fluorescens* yields a mutant with excellent enantioselectivity and activity for the kinetic resolution of a chiral building block. *ChemBioChem*, 7, 805–809, 2006.
99. Henke, E. et al., A molecular mechanism of enantiorecognition of tertiary alcohols by carboxylesterases, *ChemBioChem*, 4, 485, 2003.
100. Koga, Y. et al., Inverting enantioselectivity of *Burkholderia cepacia* KWI-56 lipase by combinatorial mutation and high-throughput screening using single-molecule PCR and *in vitro* expression, *J. Mol. Biol.*, 331, 585, 2003.
101. Bornscheuer, U.T. and Kazlauskas, R.J., Catalytic promiscuity in biocatalysis: using old enzymes to form new bonds and follow new pathways, *Angew. Chem. Int. Ed.*, 43, 6032, 2004.
102. Aharoni, A. et al., The “evolvability” of promiscuous protein functions, *Nat. Genet.*, 37, 73, 2004.
103. van Kampen, M.D. and Egmond, M.R., Directed evolution: from a staphylococcal lipase to a phospholipase, *Eur. J. Lipid Sci. Technol.*, 102, 717, 2000.
104. Kauffmann, I. and Schmidt-Dannert, C., Conversion of *Bacillus thermocatenulatus* lipase into an efficient phospholipase with increased activity towards long-chain fatty acyl substrates by directed evolution and rational design, *Protein Eng.*, 14, 919, 2001.
105. Joerger, R.D. and Haas, M.J., Alteration of chain length selectivity of a *Rhizopus delemar* lipase through site-directed mutagenesis, *Lipids*, 29, 377, 1994.

6 Flavin-Containing Oxidative Biocatalysts

Marco W. Fraaije and Willem J.H. van Berkel

CONTENTS

6.1	Introduction	181
6.2	General Properties of Flavin-Containing Oxidative Biocatalysts	181
6.3	Flavoprotein Oxidases.....	183
6.4	Carbohydrate Oxidases	185
6.5	Alcohol Oxidases.....	187
6.6	Amine Oxidases.....	188
6.7	Sulphydryl Oxidases.....	190
6.8	Flavoprotein Monooxygenases	191
6.9	Aromatic Hydroxylases.....	192
6.10	Heteroatom Monooxygenases.....	193
6.11	Two-Component Monooxygenases.....	194
	References	195

6.1 INTRODUCTION

Flavoenzymes are able to catalyze a remarkably wide variety of oxidative reactions such as regio- and enantioselective monooxygenations and highly regiospecific oxidations. Such reactions are often difficult, if not impossible, to be achieved using chemical approaches. Due to their regio- and enantioselectivity and catalytic efficiency, these enzymes have been shown to be highly valuable biocatalysts for the synthesis of a variety of fine chemicals. Until a decade ago, only a scarce number of flavin-dependent enzymes had been cloned and over-expressed, which limited the number of flavoenzyme-based biocatalytic applications. However, in recent years a large number of novel flavin-containing biocatalysts have been discovered and new biocatalytic processes have been developed based on oxidative flavoenzymes.

6.2 GENERAL PROPERTIES OF FLAVIN-CONTAINING OXIDATIVE BIOCATALYSTS

In nature, many oxidative reactions are carried out by flavin-dependent oxidoreductases [1]. These enzymes typically harbour a flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) molecule as organic cofactor (Figure 6.1). By varying the direct protein environment around the flavin, thereby modulating the redox properties of the organic cofactor, evolution has created an immense set of redox and nonredox active flavoenzymes. Well-known examples are flavin-containing dehydrogenases and oxidases that are able to oxidize organic substrates.

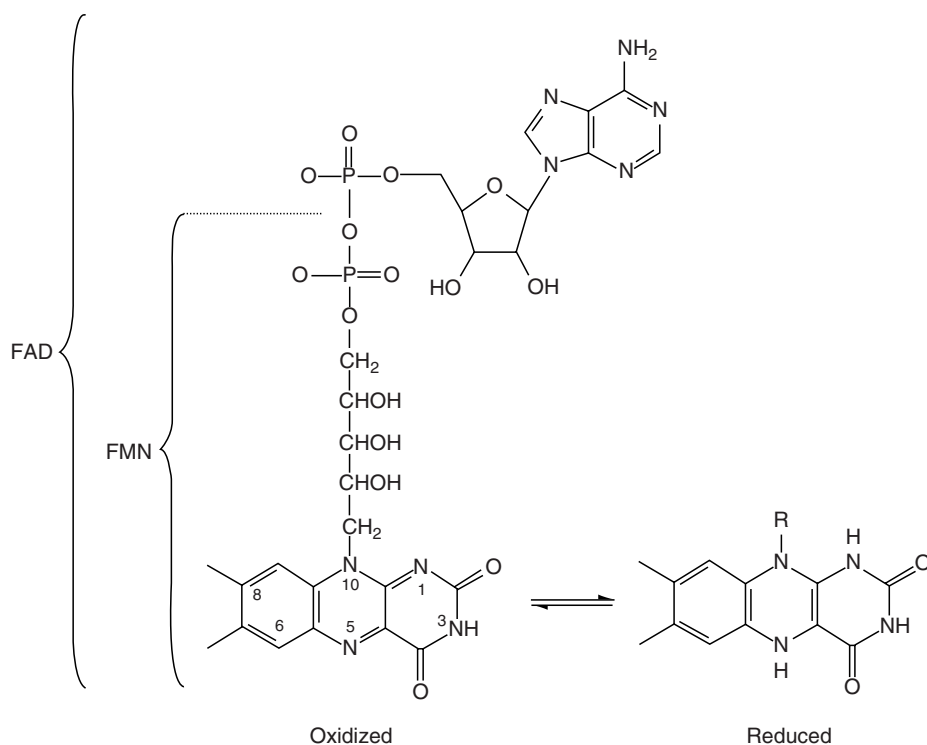


FIGURE 6.1 Structural formulas of the flavin cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).

Electron transfer is also mediated by flavoproteins and light production can be catalyzed by flavoenzymes. A large number of flavoenzymes are also able to use molecular oxygen as substrate, enabling oxyfunctionalization of hydrocarbons through, for example, Baeyer–Villiger oxidations or epoxidations. In fact, flavins are the only organic cofactors that are able to utilize molecular oxygen for oxygenation reactions. Other known oxygenating cofactors always depend on a metal ion for their reactivity. Studies on oxidative flavoenzymes during the past decades have shown that these enzymes display an astonishing variability in the type of reactions catalyzed (Figure 6.2). Such a flexibility of using molecular oxygen for different types of reactions while retaining a high degree of enantio- and/or regioselectivity has so far not been encountered in any other enzyme family [2]. The flexibility in reactivity is also reflected in the diverse physiological processes in which these enzymes are involved: regulating protein folding [3], xenobiotics metabolism in humans [4], biosynthesis of toxins [5], drug activation [6], plant growth regulation [7], and pollutant degradation by microorganisms [8].

Enantio- and regioselective oxygenations and oxidations are difficult to achieve by chemical means, while these reaction types can lead to valuable fine chemicals. Chemical synthesis routes that catalyze selective oxygen insertion or oxidation reactions often involve tedious and costly blocking and deblocking steps and are catalyzed by heavy metals. Therefore, flavin-dependent monooxygenases and oxidases represent highly attractive alternatives as these biocatalysts are able to catalyze a huge variety of monooxygenation and oxidation reactions while exhibiting a remarkable selectivity. Exploitation of these biocatalysts affords effective environment-friendly synthesis routes (“green chemistry”) that have several advantages

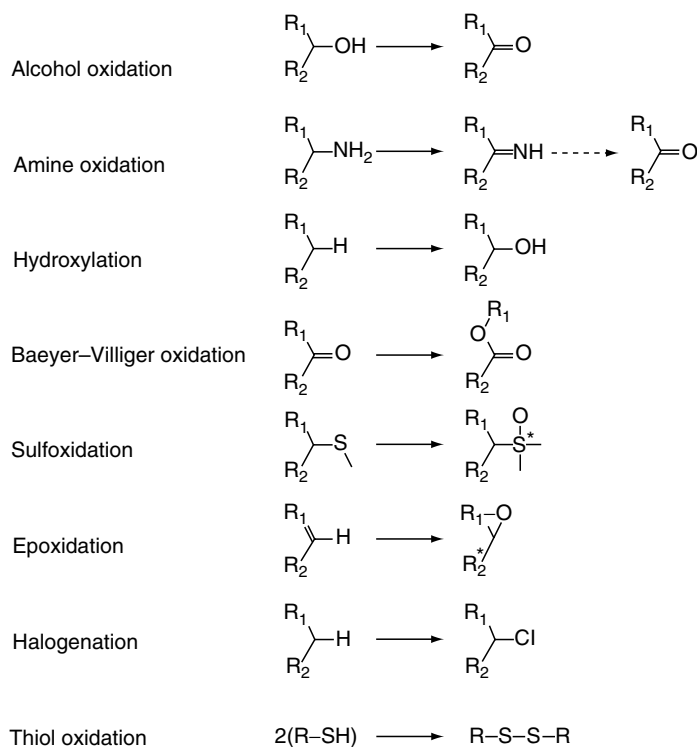


FIGURE 6.2 Example reaction types catalyzed by flavin-dependent oxidoreductases.

over chemical routes. This includes the renewability of the catalyst, reduced formation of by-products thanks to fewer side reactions, and the possibility to use mild process conditions. Except for synthesis purposes, flavin-containing enzymes can also be applied in sensing applications.

This review will give an overview on the availability and biocatalytic potential of several types of flavoenzymes of two classes: oxidases and monooxygenases.

6.3 FLAVOPROTEIN OXIDASES

Flavoprotein oxidases are enzymes that have the potential to be highly valuable for biotechnological processes as they are able to use molecular oxygen as a clean and cheap oxidant while they do not require expensive coenzymes. An increasing number of oxidases have been identified, cloned, and overexpressed in the past few years (see Table 6.1). The discovery of these novel oxidases has also revealed a number of new oxidase-linked reactivities. Not only are relatively simple alcohol or amine oxidations carried out by flavin-containing oxidases, but more chemically demanding reactions are also mediated by these enzymes. For example, vanillyl-alcohol oxidase is able to cleave an ether bond [9], while prenylcysteine oxidase (EC 1.8.3.5) is able to break a thioether bond [10]. Another striking example of the delicate catalytic power of flavoprotein oxidases is the plant enzyme (*S*)-reticuline oxidase (EC 1.21.3.3), which catalyzes the regio- and stereospecific oxidative cyclization of benzophenanthrodine alkaloids [11]. Also the biosynthesis of cannabinoids was recently shown to depend on a similar plant oxidase [12]. The common denominator for oxidases is the fact that upon

TABLE 6.1
Cloned Flavoprotein Oxidases That Are Relevant for Biocatalysis

Enzymes	Year of Cloning	EC Number	Structure/Cofactor	Origin
Carbohydrates				
Glucose oxidase	1990	1.1.3.4	+/-	f
Hexose oxidase	1997	1.1.3.5	-/+	a
Lactose oxidase	2001	1.1.3.x	-/+	f
Sorbitol/xylitol oxidase	1998/2000	1.1.3.41	-/+	b
D-Gluconolactone oxidase	2004	1.1.3.x	-/+	f
Pyranose oxidase	1996	1.1.3.10	+/+	f
Aliphatic alcohols				
Alcohol oxidase	1985	1.1.3.13	-/-	y
Isoamyl alcohol oxidase	2000	1.1.3.x	-/+	f
Long-chain alcohol oxidase	2000/2004	1.1.3.20	-/-	y/p
Cholesterol oxidase	1986	1.1.3.6	+/-, +	b
Aromatic alcohols				
Vanillyl-alcohol oxidase	1998	1.1.3.38	+/+	f
Aryl-alcohol oxidase	1999	1.1.3.7	-/-	f
Amines				
D-Amino acid oxidase	2004/1997/2002	1.4.3.1/1.4.3.3 1.4.3.19	+/-	y/b
L-Amino acid oxidase	1990/2003/2003/1988	1.4.3.2/1.4.3.11 1.4.3.14/1.4.3.16	+/-	f/y/b
Monoamine oxidase	1995	1.4.3.4	+/+	f
Fructosyl amine oxidase	1997/2002/2005	1.5.3.x	-/+	f/b/y
Others				
Sulfhydryl oxidase	2000	1.8.3.2	+/-	y/f
NADH oxidase (H ₂ O forming)	1982	1.6.3.x	-/-	b

a, algae; b, bacterial; f, fungal; y, yeast; p, plant; +, crystal structure; -, no crystal structure; +, covalent flavin; -, noncovalent flavin.

oxidation of the organic substrate, the reduced flavin cofactor transfers the electrons to molecular oxygen, yielding hydrogen peroxide:



An increasing number of oxidases is produced and successfully applied for industrial purposes. In the past, only three specific oxidases have been extensively used: glucose oxidase (e.g., for biosensing and gluconic acid production), cholesterol oxidase (e.g., for clinical assays), and D-amino acid oxidase (e.g., for synthesis of antibiotics). The biocatalytic exploitation of oxidases was mainly restricted by availability. Only in recent years has the number of available (recombinant) oxidases increased significantly.

Many of the newly discovered oxidases contain a covalently bound FAD as cofactor (Table 6.1). The physiological rationale for covalent cofactor binding is still not well understood. Nevertheless, the advantage of covalent coupling of a cofactor is multifold. One major and obvious advantage is that cofactor dissociation is prevented, which would lead to loss of activity. In fact, efficient commercial usage of D-amino acid oxidase and glucose oxidase is frequently hampered by the fact that these enzymes can lose the flavin cofactor under stress conditions. Except for saturating the enzyme active site, formation of a covalent bond can

also have beneficial effects on the reactivity of the cofactor. For vanillyl-alcohol oxidase it was shown that covalent flavinylation is an autocatalytic process [13,14], and that the covalent histidyl–FAD bond increases the redox potential of the flavin cofactor, resulting in a tenfold increase in enzyme activity [15]. Furthermore, covalent linkage may well add significantly to the protein stability as the additional covalent bond has a similar stabilizing effect to that found in intramolecular disulfide bridges [16].

Another striking observation is the fact that most discovered oxidases are of fungal origin. Only a limited number of oxidases have been found in bacteria while archaeobacterial representatives are really scarce. The sporadic occurrence of oxidases in (archaeo)bacterial genomes prevents effective mining of sequenced genomes. The low abundance of oxidases in nature might reflect that they are energy-wasting enzymes as they shuttle the generated electrons directly to molecular oxygen without exploiting the respiratory chain. As a result, most oxidoreductases in bacteria have evolved in such a way that they are able to utilize alternative electron acceptors. In fact, it is thought that many fungal oxidases serve a competitive role, instead of being part of catabolic routes. By producing hydrogen peroxide from molecular oxygen, microbes competing for the same nutrients are eliminated. Furthermore, several fungal oxidases are involved in an extracellular cascade catalytic machinery, producing hydrogen peroxide needed for the degradation of lignin by peroxidases. The low abundance of oxidases in archaeobacteria appears logical as these microbes typically grow in anoxic conditions. In fact, a known archaeal oxidase, NADH oxidase, appears to fulfill a detoxifying role by scavenging molecular oxygen at the expense of NADH. This oxidase might have biocatalytic relevance as it can be used to regenerate NAD^+ from NADH [17].

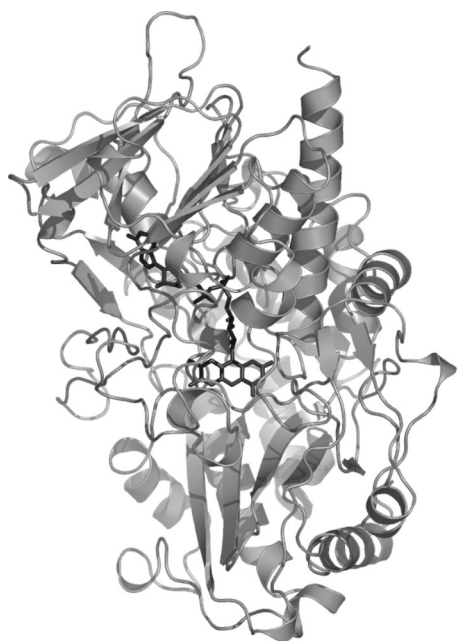
6.4 CARBOHYDRATE OXIDASES

Glucose oxidase from *Aspergillus niger* is the most widely employed oxidase. Since decades it has been used for biosensing, bleaching, bread improvement, and as oxygen scavenger [18]. A typical biocatalytic application is the use of (isolated) enzyme to produce gluconic acid. Illustrative for its wide applicability is the number of patents, which include the use of glucose oxidase, which is well above 5000. As glucose oxidase has been the subject of biochemical studies for a long time, many of its molecular properties are well described, including its crystal structure (Figure 6.3) [19,20] and catalytic mechanism [21].

Glucose oxidase from *A. niger* is a homodimeric FAD-containing glycoprotein. It catalyzes the oxidation of β -D-glucose by molecular oxygen to D-glucono-1,5-lactone, which subsequently hydrolyzes spontaneously to gluconic acid. The enzyme obeys a ping-pong bi-bi kinetic mechanism, which implies that the glucono- δ -lactone product generated in the reductive half-reaction dissociates before the reduced enzyme reacts with oxygen to regenerate the oxidized enzyme. It has been proposed that β -D-glucose is oxidized by a concerted transfer of a proton from its C1-hydroxyl to an active site histidine and a direct hydride transfer from its C1 position to flavin-N5 [22].

Glucose oxidase has a narrow specificity with electron-donor substrates. Numerous derivatives of β -D-glucose are converted, but the physiological substrate is by far the best. In contrast, a large number of substrates can replace molecular oxygen in the oxidative half-reaction. In addition to benzoquinones and naphthaquinones, which act as efficient two-electron acceptors, the enzyme is active with many one-electron oxidants including metal-ion complexes. These mediators are widely applied in glucose oxidase electrodes [21].

Only recently several alternative flavin-dependent oxidases acting on carbohydrates have been discovered. One of these oxidases, hexose oxidase from seaweed, is presently produced at a large scale by Danisco and has shown to be a valuable biocatalyst. Production of this



Glucose oxidase



Vanillyl-alcohol oxidase



p-Hydroxybenzoate hydroxylase



Phenylacetone monooxygenase

FIGURE 6.3 Crystal structures of glucose oxidase, vanillyl-alcohol oxidase, *p*-hydroxybenzoate hydroxylase, and phenylacetone monooxygenase (PDB files 1GAL, 1VAO, 1PHH, and 1W4X, respectively). The flavin cofactor is shown in black and is shown in a similar orientation in each figure. Note that in vanillyl-alcohol oxidase also the covalent histidyl linkage is shown.

oxidase was only feasible after developing an efficient microbial expression system for this plant enzyme [23]. Hexose oxidase is able to oxidize a variety of carbohydrates including D-glucose, D-galactose, maltose, cellobiose, and lactose. By this, it contrasts with the restricted substrate specificity of glucose oxidase. Another striking difference is the mode of flavin cofactor binding. While in glucose oxidase the FAD cofactor is dissociable, hexose oxidase contains a covalently bound FAD cofactor, attached to a histidine residue [23].

Recently, yet another carbohydrate oxidase has become available for biocatalytic applications: pyranose oxidase. This oxidase originates from fungi and, like hexose oxidase, contains a covalently bound FAD cofactor. It has been shown that this fungal enzyme can be overexpressed in *Escherichia coli* [24]. In 2004 the crystal structure of pyranose oxidase was elucidated showing some structural resemblance with glucose oxidase [25,26]. Nevertheless, the active sites of these fungal oxidases differ to a great extent. This agrees with the fact that pyranose oxidase selectively oxidizes D-glucose and related carbohydrates at the C2 position, while glucose oxidase and hexose oxidase oxidize exclusively the C1-hydroxyl moiety. As a consequence, a totally novel product spectrum can be obtained using pyranose oxidase. For example, the oxidation of D-glucose by pyranose oxidase yields 2-keto-D-glucose, which can serve as precursor for the synthesis of, for example, D-fructose [27] or the antibiotic cortalcosterone [28]. To get an impression of the biocatalytic potential of pyranose oxidase, the reader is referred to a review by Giffhorn [29]. The thermostability and catalytic efficiency of *Peniophora gigantea* pyranose oxidase has been improved by a dual site-directed mutagenesis and molecular evolution approach [30].

The above-mentioned carbohydrate oxidases preferably act on monosaccharides. Recent reports on newly discovered oxidases have changed this situation. A fungal lactose oxidase and some plant oxidases have been found to accept di- and polysaccharides [31,32]. Lactose oxidase catalyzes the production of lactobionic acid, which can be used as food additive and in a range of other applications [33]. In addition, bacterial oxidases acting on alditols (xylitol and sorbitol oxidase) and a fungal oxidase acting on aldonoalactones (D-gluconolactone oxidase) were identified and expressed in recent years [34–36].

6.5 ALCOHOL OXIDASES

Several flavoprotein oxidases have been described that are active with short-chain aliphatic alcohols. The most well-known enzymes are the methanol oxidases from yeast [37]. For oxidation of long-chain alcohols (C₄–C₂₂), other yeast and also plant oxidases have been identified and expressed as recombinant enzymes [38–40]. Recently, a fungal oxidase has been described that is primarily active on a branched-chain alcohol: isoamyl alcohol [41].

FMN-dependent alcohol oxidases that are active with 2-hydroxy acids are also widespread [42,43]. Like other secondary alcohol oxidases [44], these enzymes can be applied in a coupled enzyme approach for the development of deracemization processes. Thus, glycolate oxidase from spinach was used for the enantioselective oxidation of various *rac*-2-hydroxy acids, yielding the corresponding 2-keto acid and the nonconverted (*R*)- α -hydroxy acid. The 2-keto acid was then further converted using D-lactate dehydrogenase and formate dehydrogenase (for NADH regeneration), yielding the (*R*)- α -hydroxy acid in high enantiomeric excess and high yield [45]. In another chemoenzymatic approach (see also below), sodium borohydride was used to deracemize DL-lactate using L-lactate oxidase as the enantioselective enzyme [46].

Lactate is a substrate of great interest in clinical and sport medicine. Therefore, lactate oxidase-based biosensors are being developed for continuous monitoring of the condition of patients and athletes. These miniaturized devices are based on the action of immobilized lactate oxidase and electrochemical detection [47,48].

Alcohol oxidases that have evolved to oxidize cholesterol and related steroids have been found in several bacteria. These 3 β -hydroxysteroid oxidases, more commonly referred to as cholesterol oxidases, are bifunctional enzymes because they also catalyze the isomerization of the initially formed 5-cholesten-3-one into 4-cholesten-3-one.

Two types of cholesterol oxidases have been described containing a dissociable (I) or covalently bound (II) FAD cofactor [49]. The type I form of the enzyme contains a His and Asn that are critically involved in substrate oxidation and a Glu that acts as a base in the isomerization reaction. The type II cholesterol oxidase has a more hydrophilic active site and contains an Arg that replaces the His–Asn pair in the type I reaction. The type II enzyme has a higher redox potential and thus a stronger oxidation power.

Cholesterol oxidase can be exploited as clinical reagent [50] and as insecticide [51]. The clinical use in cholesterol serum determinations is based on the colorimetric detection of the enzymatically produced hydrogen peroxide. The enzyme is also active with other sterols and several structurally unrelated alcohols [52]. With regard to the substrate specificity, it must be stressed that the catalytic efficiency of cholesterol oxidase is sensitive to the activity of the substrate in the mixed phase. Truncation of an active site loop appeared to be important for movement of cholesterol from the lipid bilayer [53]. Mutation of a wide variety of specific amino acid residues altered the catalytic efficiency of the type I enzyme without changing its substrate specificity [54].

Two flavoprotein oxidases that efficiently convert aromatic alcohols are available in recombinant form. Studies on vanillyl-alcohol oxidase revealed that this enzyme is very specific toward phenolic substrates, while it displays an exceptional flexibility in oxidation reactivity. In addition to alcohol oxidations, amine oxidation, dehydrogenation, demethylation, and hydroxylation reactions can be catalyzed [9,55]. Elucidation of the vanillyl-alcohol oxidase structure (Figure 6.3) confirmed that the phenolic moiety of the substrate is a prerequisite for binding and activation of the substrate [56]. It was also shown that vanillyl-alcohol oxidase catalysis involves the formation of quinone methide product intermediates [57]. These electrophilic compounds are attacked by water in the enzyme active site, leading, for instance, to the production of chiral 1-(4'-hydroxyphenyl)alcohols [58]. By tuning the water activity during turnover, the relative amount of hydroxylated or alkenic product could be modulated [59]. Furthermore, the crystallographic data enabled the rational design of an enzyme variant that displays an inverse enantioselectivity toward the hydroxylation of 4-ethylphenols [60]. Vanillyl-alcohol oxidase can also be used for the production of natural vanillin [61]. Using molecular evolution it was possible to turn the suicide inhibitor creosol into a vanillin precursor [62].

Aryl-alcohol oxidase from *Pleurotus eryngii* was cloned and expressed recently [63]. This monomeric enzyme is related in structure to glucose oxidase. Substrate profiling studies revealed that aryl-alcohol oxidase is complementary to vanillyl-alcohol oxidase by accepting nonphenolic aromatic substrates [64]. The enzyme also oxidizes unsaturated aliphatic primary alcohols [65].

6.6 AMINE OXIDASES

The ability of certain flavoprotein oxidases to oxidize amines has also attracted biocatalytic interest. A classic example concerns D-amino acid oxidase, which catalyzes the stereospecific deamination of D-amino acids to the corresponding α -keto acids along with the production of ammonia and hydrogen peroxide through an imino acid intermediate (Figure 6.2). D-amino acid oxidase has a broad substrate specificity [66] and is used on an industrial scale for the synthesis of glutaryl-7-aminocephalosporinic acid from the natural antibiotic cephalosporin C [67]. This reaction is part of a two-enzyme process toward the production of

7-aminocephalosporinic acid, a key intermediate in the production of semisynthetic cephalosporin antibiotics. The enzyme is also used in biosensors and for the production of α -keto acids [68].

The relationships between structure and function of pig kidney and yeast D-amino acid oxidase have been studied in great detail. Both enzymes are homodimers, with each subunit containing a relatively weakly bound FAD [69]. The mammalian enzyme is more thermostable, but is a less efficient biocatalyst. For industrial applications, the enzyme is used in immobilized form, resulting in improved biocatalyst stability [70]. To protect the enzyme from hydrogen peroxide inactivation, methionine residues susceptible to oxidation were removed by site-directed mutagenesis [71].

L-amino acid oxidase is the enantiomerically opposite of D-amino acid oxidase. It is a homodimeric FAD-dependent glycoprotein and present at significantly high concentrations in snake venoms [72,73]. Snake venom L-amino acid oxidase has a more narrow substrate specificity than D-amino acid oxidase, exhibiting a marked preference for bulky hydrophobic amino acids. Comparison of the crystal structures of L- and D-amino acid oxidase revealed a mirror-symmetrical relationship between the two substrate binding sites, which facilitates the enantiomeric selectivity while preserving a common arrangement of the atoms involved in catalysis [72]. Several recombinant forms of bacterial L-amino acid oxidases have recently been described [74,75]. These enzymes differ strongly in substrate preference. The enzyme from *Rhodococcus opacus* exhibits a much broader substrate specificity than the ophidian L-amino acid oxidase and is an attractive catalyst for enzymatic synthesis.

Recently, it was shown that D- and L-amino acid oxidase are valuable biocatalysts for deracemization of α -amino acids [76,77]. By performing *in situ* chemical reduction of the formed imine product, one of the two amino acid enantiomers was converted in the other enantiomer. This elegant and effective combination of an enzymatic oxidation and chemical reduction demonstrates the versatile applicability of these types of enzymes. The chemoenzymatic system can also be used to interconvert diastereomeric amino acids bearing more than one stereocenter [78].

Monoamine oxidase is a flavoenzyme that is closely related in structure to L-amino acid oxidase [79]. In vertebrates, this 8 α -S-cysteinyl-FAD-containing enzyme is bound to the mitochondrial outer membrane where it catalyzes the oxidative deamination of neurotransmitters and biogenic amines. The human enzyme, which occurs in two isoforms (A and B), is involved in a large number of diseases, and is an important target for antidepressant and neuroprotective drugs. Phylogenetic analysis has indicated that monoamine oxidase was already present in early eukaryotes, but that its gene was lost in worm, fly, plant, and yeast before gene duplication in terrestrial vertebrates occurred [80]. A single monoamine oxidase gene seems to be present in aquatic vertebrates, whereas a different situation was found with amphibians [81].

Another eukaryotic monoamine oxidase was discovered in *A. niger* [82]. This enzyme has a considerably higher turnover number on many aliphatic and aromatic amines than the mammalian isoforms but is not active with biogenic amines [83]. *A. niger* monoamine oxidase has been exploited for the deracemization of chiral primary amines. To enhance the efficiency of this process, *in vitro* evolution was used to convert the fungal enzyme into a more competent biocatalyst [84]. Through this approach, it was also possible to enhance the activity with secondary amines [85].

The development of high-level expression systems for producing recombinant human liver monoamine oxidases in the methylotrophic yeast *Pichia pastoris* [86,87] has facilitated structure–function relationship studies and provided much insight into the mode of binding of clinically used drug inhibitors [88–90]. Despite this, the mechanism of substrate oxidation remains controversial [91]. Based on the active site topology and previous structure activity

studies, a concerted polar nucleophilic mechanism for monoamine oxidase catalysis, involving substrate–flavin adduct formation, was proposed [92]. Very recently, however, a tyrosyl radical, suggested to be the key missing link in support of the single electron transfer mechanism, was identified in monoamine oxidase A [93].

Monoamine oxidases have a broad substrate specificity and can be used in several applications. As noted above, these enzymes are useful for the deracemization of chiral amines. Another application concerns the immobilization of different monoamine oxidase isoforms to create online immobilized enzyme reactors. These systems can be used for the rapid identification of monoamine oxidase inhibitors in complex chemical and biological mixtures [94]. Because altered levels of monoamine oxidase activity are associated with many neurological and psychiatric diseases, there is also a need for imaging the enzyme in intact cells and tissues. Toward this goal, a fluorogenic probe for monoamine oxidase was developed that can act as an irreversible redox switch [95]. In short, this switch is based on the enzymatic oxidation of the ethylamino group of an aminocoumarin substrate, affording the aldehyde product, which subsequently undergoes spontaneous intramolecular condensation, furnishing an indole moiety that alters the fluorescence profile.

Relatively newly discovered flavoprotein oxidases that act on an amine moiety are the fructosyl amine oxidases [96]. These fungal enzymes are also called amadoriases as they act on Amadori products: glycated proteins. As for some of the above-mentioned oxidases, these were again found to contain a covalently bound FAD cofactor. Their role in fungal physiology is still obscure. It has been shown that these enzymes facilitate growth when using fructosyl-amino acids as nitrogen source [97]. Furthermore, a recent report suggests a role in recycling aged (i.e., glycated) amino acids in fungal cells [98]. The fructosyl amine oxidases have been studied for their putative value for detecting glycated proteins in blood samples. This would enable efficient clinical diagnosis of diabetes mellitus. Amadoriases may also be useful to eliminate Maillard reaction products. To improve the catalytic properties of these enzymes, molecular evolution has successfully been applied, resulting in thermostable variants with improved affinity for glycated amino acids [99].

6.7 SULFHYDRYL OXIDASES

Sulfhydryl oxidases represent a relatively new group of flavoprotein oxidases [100]. These enzymes catalyze the insertion of disulfide bonds into proteins and/or the oxidation of small-molecular weight thiols. Flavin-containing sulfhydryl oxidases are present in many eukaryotic species, where they are targeted to different subcellular compartments and specific oxidation reactions. Most sulfhydryl oxidases contain a small FAD-binding domain with a unique fold, which can be found as a single-domain protein (Erv/ALR family) or fused to an N-terminal thioredoxin domain in the QSOX family. Some of the single-domain sulfhydryl oxidases are *in vivo* linked to the function of protein disulfide isomerase and a source of disulfide bonds for oxidative protein folding in the cell [101]. *In vitro*, however, single-domain sulfhydryl oxidases are only active with small-molecular weight thiols such as dithiothreitol and reduced glutathione.

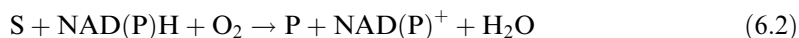
Chicken sulfhydryl oxidase is presently the most well-documented flavin-dependent biocatalyst to introduce disulfide bridges directly into a wide range of unfolded reduced proteins and peptides [100]. This QSOX-type enzyme is a heavily glycosylated homodimer, with each subunit containing a noncovalently bound FAD and a redox-active disulfide. The catalytic efficiency of chicken sulfhydryl oxidase is not strongly dependent on the size or charge of the protein substrate. However, with RNase as a protein substrate, the inclusion of protein disulfide isomerase is required for the efficient recovery of native disulfide bonds.

Sulfhydryl oxidases may be applied in breadmaking by improving the strength and handling properties of the dough [102]. Removal of low-molecular weight thiols such as reduced glutathione during mixing may prevent their participation in the thiol–disulfide exchange reactions, which result in the depolymerization of the gluten proteins and thereby reduce the dough elasticity and increase its extensibility. Moreover, the hydrogen peroxide generated may be used in a peroxidase-mediated reaction to catalyze the oxidative gelation of the wheat flour pentosans. A similar mechanism of peroxidase activation can account for the improving effect in breadmaking by application of different carbohydrate oxidases [103,104].

Apart from all the above-mentioned oxidases, other flavin-containing oxidases are also available in recombinant form. Most of these enzymes, like sarcosine oxidase [105], polyamine oxidase [106,107], nitroalkane oxidase [108,109], and γ -*N*-methylaminobutyrate oxidase [110], have a limited substrate specificity. Nevertheless, knowledge about their structure and function will contribute to the future development of flavin-containing oxidative biocatalysts.

6.8 FLAVOPROTEIN MONOOXYGENASES

Flavoprotein monooxygenases catalyze the insertion of one atom of dioxygen into the substrate according to the following scheme:



Several types of flavin-dependent monooxygenases have been discovered during the past few decades. While some of these enzymes have been isolated from eukaryotes (e.g., fungi and yeasts), the best-studied flavin-dependent monooxygenases are from bacterial origin. In fact, almost all cloned flavoprotein monooxygenases are from bacteria. This is in contrast with the situation for oxidases where many eukaryotic representatives have been cloned and over-expressed. Also different from the oxidases is the observation that all characterized monooxygenases contain a flavin cofactor that is dissociable. This might indicate that for catalytic functioning the flavin should not be restricted in mobility. This is in line with the fact that for some monooxygenases the flavin has been found to adopt multiple orientations within the protein structure during the catalytic cycle [111,112]. This is in contrast with the oxidases in which the active site is typically buried in the protein structure and can be regarded as relatively rigid.

Roughly, three classes of flavoprotein monooxygenases can be identified (Table 6.2). The first entails hydroxylases that typically act on aromatic compounds; the prototype for these monooxygenases is *p*-hydroxybenzoate hydroxylase (PHBH). The second comprises monooxygenases that can catalyze Baeyer–Villiger oxidations and heteroatom oxidations. In this review we refer to this class as heteroatom monooxygenases. The first structure of a member of this class of monooxygenases has just been elucidated [113]. The third can be defined as a group of monooxygenases that depend on two different subunits: the two-component monooxygenases. This review only aims at illustrating the biocatalytic potential of flavoprotein monooxygenases. The following sections describe the catalytic versatility of some specific monooxygenases that have been cloned and described in the literature. However, this only reflects part of the industrial applicability of such oxidative enzymes. For example, at Lonza several industrial processes rely on the use of isolated bacterial strains that exhibit specific hydroxylation activities [114]. While molecular details of these biocatalysts might not be known, several of them might well represent flavoproteins.

TABLE 6.2
Cloned Flavoprotein Monooxygenases That Are Relevant for Biocatalysis

Enzymes	Year of Cloning	EC Number	Structure	Origin
Aromatic hydroxylases				
4-Hydroxybenzoate 3-hydroxylase	1988	1.14.13.2	+	b
Phenol hydroxylase	1992	1.14.13.7	+	y
2-Hydroxybiphenyl 3-monooxygenase	1997	1.14.13.44	—	b
Baeyer–Villiger monooxygenases				
Phenylacetone monooxygenase	2004	1.14.13.92	+	b
Cyclohexanone monooxygenase	1988	1.14.13.22	—	b
Cyclopentanone monooxygenase	2002	1.14.13.16	—	b
Cyclododecanone monooxygenase	2001	1.14.13.x	—	b
Steroid monooxygenase	1999	1.14.13.54	—	b
4-Hydroxyacetophenone monooxygenase	2001	1.14.13.84	—	b
Two-component monooxygenases				
Tryptophan 7-halogenase	2000	1.14.14.x	+m	b
Phenol 2-monooxygenase	2003	1.14.14.x	+r	b
Styrene monooxygenase	1996	1.14.14.x	—	b
Phenazine 1-carboxylic acid monooxygenase	2001	1.14.14.x	—	b
Dibenzothiophene monooxygenase	1994	1.14.14.x	—	b

b, bacterial; y, yeast; m, monooxygenase component; r, reductase component.

6.9 AROMATIC HYDROXYLASES

The most extensively studied class of flavoprotein monooxygenases is the group of aromatic hydroxylases that shows sequence homology with PHBH. The three-dimensional structure of this prototype monooxygenase was already elucidated in 1979 (Figure 6.3) [115,116] and triggered several groups to study its molecular details [111,117]. The folding topology of PHBH is shared by several flavin-containing biocatalysts including glucose oxidase, cholesterol oxidase, D-amino acid oxidase, monoamine oxidase, and phenol hydroxylase [118].

Flavoprotein aromatic hydroxylases contain a noncovalently bound FAD cofactor and depend on NADH or NADPH for activity. Most representatives have many catalytic properties in common and their overall reactions can be divided into two half-reactions [2]. In the reductive half-reaction, the FAD cofactor becomes reduced and the NADP⁺ coenzyme is released. In the oxidative half-reaction, the reduced flavin reacts with molecular oxygen, yielding the flavinhydroperoxide oxygenation species. Protonation of the distal oxygen of the peroxide moiety increases the electrophilic reactivity of the flavin peroxide and facilitates its attack on the nucleophilic carbon center of the substrate. Rapid reaction studies with substrate analogs have indicated that the aromatic product is initially formed in its keto isomeric form, which isomerizes to give the energetically favored dihydroxy isomer [2].

Flavoprotein aromatic hydroxylases display a subtle mechanism of substrate recognition [119]. For efficient turnover, the substrate should act as an effector to induce flavin reduction by NAD(P)H. Moreover, upon binding to the enzyme active site, the substrate must become activated to allow hydroxylation and to prevent the unproductive decomposition of the flavinhydroperoxide [111]. Studies from PHBH variants, created by site-directed mutagenesis, have shown that a great number of strictly conserved amino acid residues are involved in substrate binding and activation [117]. As a result, these monooxygenases typically display a narrow substrate specificity and perform highly regioselective hydroxylations [120–122].

There are a few flavin-dependent aromatic hydroxylases with a rather relaxed substrate specificity. One of these enzymes, phenol hydroxylase, was originally obtained from the yeast *Trichosporon cutaneum*, but is now available in recombinant form [123]. Another aromatic hydroxylase with a rather broad substrate spectrum concerns 2-hydroxybiphenyl 3-monooxygenase. This enzyme is induced in *Pseudomonas azelaica* HBP1, and is active with a wide range of 2'-substituted phenols [124]. The 2-hydroxybiphenyl 3-monooxygenase gene has been cloned in *E. coli* and the recombinant strain was used as a whole-cell biocatalyst for the production of 2-substituted catechols [125,126]. Molecular evolution has been used to improve the catalytic scope and performance of 2-hydroxybiphenyl 3-monooxygenase. In this way it was possible to increase the hydroxylation efficiency [127] and extend the substrate spectrum [128,129].

6.10 HETEROATOM MONOOXYGENASES

The second flavoprotein monooxygenase class entails enzymes that share sequence homology with phenylacetone monooxygenase. The crystal structure of this enzyme (Figure 6.3) was recently solved and represents the first structure of a Baeyer–Villiger monooxygenase [113]. Most heteroatom monooxygenases contain a tightly bound FAD cofactor and are typically dependent on NADPH as electron donor [130]. Representatives of this class have been found to catalyze a relatively broad range of asymmetric oxygenation reactions with high enantio- or enantiotoposelectivity. In addition to Baeyer–Villiger reactions, a range of other oxidation reactions can be catalyzed including sulfoxidations, amine hydroxylations, organoboron oxidations, and epoxidations. Based on genome analysis it has been found that many bacteria and fungi contain heteroatom monooxygenases that preferentially act as Baeyer–Villiger monooxygenases [131]. Plant and vertebrate genomes are rich in heteroatom monooxygenases that preferentially act on soft nucleophilic heteroatoms (e.g., S and N) and are known as flavin-containing monooxygenases (FMOs). In humans these FMOs are involved in detoxification. Mutations in FMO genes have been shown to result in serious health problems [132].

Microbial Baeyer–Villiger monooxygenases appear to be mainly involved in the degradation of natural ketones. A number of bacterial Baeyer–Villiger monooxygenases have been extensively studied for their biocatalytic potential. Since the 1970s, cyclohexanone monooxygenase from *Acinetobacter* NCIB 9871 has been explored to a great extent with respect to its catalytic properties [133–136]. The enzyme has also been used in dynamic kinetic resolution reactions, which combine *in situ* racemization with a kinetic resolution step [137]. So far, over 100 different substrates have been described for cyclohexanone monooxygenase including ketones, aldehydes, sulfides, amines, olefins, seleno compounds, and organoboronic compounds. Also for other Baeyer–Villiger monooxygenases quite a number of substrates have been identified [130,138]. Nevertheless, each specific Baeyer–Villiger monooxygenase appears to prefer a certain class of substrates. For example, 4-hydroxyacetophenone monooxygenase efficiently converts acetophenones and benzaldehydes while it is poorly active with cyclic aliphatic ketones [130,139,140]. Therefore, it is unlikely that such enzyme activity will be detected in high-throughput fluorescence assays based on the conversion of 2-coumaroyloxyketones [141]. The typical relaxed substrate acceptance of Baeyer–Villiger monooxygenases probably reflects a common mechanistic feature of these types of enzymes. Kinetic studies have revealed that Baeyer–Villiger monooxygenases, independent from substrate binding, will form and stabilize a peroxyflavin when NADPH and oxygen are available [142]. Consequently, these enzymes will oxidize substrates that are reactive enough and are able to reach the activated flavin. Depending on the shape of the substrate binding pocket, regio- and/or enantioselective oxygenation reactions are catalyzed. This concept of selectivity tuning by preferential binding of the substrate was recently demonstrated in an enzyme redesign study

using phenylacetone monooxygenase [143]. By partial deletion of a loop that shapes the substrate binding pocket, the substrate acceptance and enantioselectivity could be significantly altered. The resulting mutant could accept substrates that are not converted by the wild-type enzyme while displaying altered enantioselectivity.

It has been found that bacterial Baeyer–Villiger monooxygenases also represent interesting targets for treating infections. Many pathogenic bacteria carry one or more Baeyer–Villiger monooxygenase genes in their genome [131]. In fact, Baeyer–Villiger monooxygenases have already been exploited as drug target for several decades to treat tuberculosis and leprosy. The corresponding mycobacteria, *Mycobacterium tuberculosis* and *M. leprae*, produce a Baeyer–Villiger monooxygenase that converts a number of commonly used antibiotics into toxic bactericidal products [144].

6.11 TWO-COMPONENT MONOOXYGENASES

The third class of flavoprotein monooxygenases consists of multicomponent enzymes. The variety in this group of monooxygenases is quite large concerning cofactor use and coenzyme specificity. Generally, these enzymes comprise two polypeptide chains: a reductase component carrying out flavin reduction and an oxygenase component oxidizing the substrate by molecular oxygen [145,146]. The reducing equivalents needed for the oxygenation are transferred from NAD(P)H to a flavin bound to the reductase component. With some reductases, a tightly bound flavin acts as extra serving-hatch of electrons [147]. Subsequently, the reduced flavin is transferred to the oxygenase component where the activation of molecular oxygen and the reaction with substrate occur. A well-known example of this monooxygenase class is 4-hydroxyphenylacetate-3-monooxygenase from *E. coli* W, consisting of a large oxygenase (59 kDa) and a small reductase (19 kDa) [148]. Another example of a two-component monooxygenase is styrene monooxygenase that has been found in a number of pseudomonads [149–152]. This enzyme is of value for biotechnological applications as it catalyzes highly enantioselective epoxidation reactions [153,154].

In addition to hydroxylation and epoxidation reactions, desulfurization reactions can also be carried out by two-component flavoprotein monooxygenases. Dibenzothiophene (DBT) desulfurization has been studied in a variety of microorganisms [155]. The genes responsible for the degradation pathway have been cloned from *Rhodococcus* sp. IGTS8 [156–158]. The removal of sulfur from the substrate requires action of two monooxygenase proteins (DszA and DszC) that are dependent on the availability of reduced FMN. Reduced FMN is generated by a reductase (DszD) that uses NADH as electron source and is related to the reductase components of 4-hydroxyphenylacetate-3-monooxygenase and styrene monooxygenase, discussed above. Biocatalytic processes using desulfurizing flavoprotein monooxygenases may find application in the desulfurization of fossil fuels [155]. Different approaches have been used to engineer strains with improved desulfurization activity. Overexpression of DszD or another flavin reductase in *E. coli* and *P. putida* enhanced the overall rate of desulfurization [159,160]. Furthermore, activities toward (highly) alkylated DBTs have been improved using a chemostat approach [161] and gene shuffling [162].

A special member of the two-component flavin-dependent monooxygenase family is tryptophan 7-halogenase [163–165]. Several bacterial enzymes have been found that are able to insert a halogen atom into an organic substrate [166]. This reaction is performed at the expense of a reduced flavin cofactor and molecular oxygen. The catalyzed halogenation reactions are typically regioselective and, as a result, these enzymes bear great potential for biocatalytic applications [167]. Recently, the crystal structure of the monooxygenase component of tryptophan 7-halogenase was elucidated [168]. Based on the binding mode of the flavin, the tryptophan substrate, and a chloride ion in the active site, an oxidative

halogenation mechanism, involving the formation and channeling of hypochlorous acid, is proposed.

In view of the need of expensive coenzymes, flavin-dependent monooxygenases are mostly applied in whole-cell systems. However, with the continuous advancements in heterologous protein expression and coenzyme regeneration, even (partially) purified monooxygenases will become increasingly attractive for the environmentally benign and cost-competitive production of high-value chemicals.

REFERENCES

1. Massey, V., The chemical and biological versatility of riboflavin, *Biochem. Soc. Trans.*, 28(4), 283–296, 2000.
2. Massey, V., Activation of molecular oxygen by flavins and flavoproteins, *J. Biol. Chem.*, 269(36), 22459–22462, 1994.
3. Suh, J.K., Poulsen, L.L., Ziegler, D.M., et al., Yeast flavin-containing monooxygenase generates oxidizing equivalents that control protein folding in the endoplasmic reticulum, *Proc. Natl. Acad. Sci. USA*, 96(6), 2687–2691, 1999.
4. Ziegler, D.M., Flavin-containing monooxygenases: enzymes adapted for multisubstrate specificity, *Trends Pharmacol. Sci.*, 11(8), 321–324, 1990.
5. Keller, N.P., Watanabe, C.M., Kelkar, H.S., et al., Requirement of monooxygenase-mediated steps for sterigmatocystin biosynthesis by *Aspergillus nidulans*, *Appl. Environ. Microbiol.*, 66(1), 359–362, 2000.
6. DeBarber, A.E., Mdluli, K., Bosman, M., et al., Ethionamide activation and sensitivity in multi-drug-resistant *Mycobacterium tuberculosis*, *Proc. Natl. Acad. Sci. USA*, 97(17), 9677–9682, 2000.
7. Zhao, Y., Christensen, S.K., Fankhauser, C., et al., A role for flavin monooxygenase-like enzymes in auxin biosynthesis, *Science*, 291(5502), 306–309, 2001.
8. Wieser, M., Wagner, B., Eberspacher, J., et al., Purification and characterization of 2,4,6-trichlorophenol-4-monooxygenase, a dehalogenating enzyme from *Azotobacter* sp. strain GP1, *J. Bacteriol.*, 179(1), 202–208, 1997.
9. Fraaije, M.W., Veeger, C., and van Berkel, W.J.H., Substrate specificity of flavin-dependent vanillyl-alcohol oxidase from *Penicillium simplicissimum*: evidence for the production of 4-hydroxycinnamyl alcohols from 4-allylphenols, *Eur. J. Biochem.*, 234(1), 271–277, 1995.
10. Digits, J.A., Pyun, H.J., Coates, R.M., et al., Stereospecificity and kinetic mechanism of human prenylcysteine lyase, an unusual thioether oxidase, *J. Biol. Chem.*, 277(43), 41086–41093, 2002.
11. Kutchan, T.M. and Dittrich, H., Characterization and mechanism of the berberine bridge enzyme, a covalently flavinylated oxidase of benzophenanthridine alkaloid biosynthesis in plants, *J. Biol. Chem.*, 270(41), 24475–24481, 1995.
12. Sirikantaramas, S., Morimoto, S., Shoyama, Y., et al., The gene controlling marijuana psychoactivity: molecular cloning and heterologous expression of delta1-tetrahydrocannabinolic acid synthase from *Cannabis sativa* L., *J. Biol. Chem.*, 279(38), 39767–39774, 2004.
13. Fraaije, M.W., van den Heuvel, R.H.H., van Berkel, W.J.H., et al., Structural analysis of flavinylation in vanillyl-alcohol oxidase, *J. Biol. Chem.*, 275(49), 38654–38658, 2000.
14. Hassan-Abdallah, A., Bruckner, R.C., Zhao, G., et al., Biosynthesis of covalently bound flavin: isolation and *in vitro* flavinylation of the monomeric sarcosine oxidase apoprotein, *Biochemistry*, 44(17), 6452–6462, 2005.
15. Fraaije, M.W., van den Heuvel, R.H.H., van Berkel, W.J.H., et al., Covalent flavinylation is essential for efficient redox catalysis in vanillyl-alcohol oxidase, *J. Biol. Chem.*, 274(50), 35514–35520, 1999.
16. Caldinelli, L., Iametti, S., Barbiroli, A., et al., Dissecting the structural determinants of the stability of cholesterol oxidase containing covalently bound flavin, *J. Biol. Chem.*, 280(24), 22572–22581, 2005.
17. Ödman, P., Wellborn, W.B., and Bommarius, A.S., An enzymatic process to α -ketoglutarate from L-glutamate: the coupled system L-glutamate dehydrogenase/NADH oxidase, *Tetrahedron Asymmetry*, 15(18), 2933–2937, 2004.

18. Wilson, R. and Turner, A.P.F., Glucose oxidase: an ideal enzyme, *Biosens. Bioelectr.*, 7(3), 165–185, 1992.
19. Hecht, H.J., Kalisz, H.M., Hendle, J., et al., Crystal structure of glucose oxidase from *Aspergillus niger* refined at 2.3 Å resolution, *J. Mol. Biol.*, 229(1), 153–172, 1993.
20. Wohlfahrt, G., Witt, S., Hendle, J., et al., 1.8 and 1.9 Å resolution structures of the *Penicillium amagasakiense* and *Aspergillus niger* glucose oxidases as a basis for modelling substrate complexes, *Acta Crystall. Section D*, 55(5), 969–977, 1999.
21. Leskovac, V., Trivic, S., Wohlfahrt, G., et al., Glucose oxidase from *Aspergillus niger*: the mechanism of action with molecular oxygen, quinones, and one-electron acceptors, *Int. J. Biochem. Cell. Biol.*, 37(4), 731–750, 2005.
22. Wohlfahrt, G., Trivi , S., Zeremski, J., et al., The chemical mechanism of action of glucose oxidase from *Aspergillus niger*, *Mol. Cell. Biochem.*, 260(1), 69–83, 2004.
23. Wolff, A.M., Hansen, O.C., Poulsen, U., et al., Optimization of the production of *Chondrus crispus* hexose oxidase in *Pichia pastoris*, *Protein Expr. Purif.*, 22(2), 189–199, 2001.
24. Vecerek, B., Maresova, H., Kocanova, M., et al., Molecular cloning and expression of the pyranose 2-oxidase cDNA from *Trametes ochracea* MB49 in *Escherichia coli*, *Appl. Microbiol. Biotechnol.*, 64(4), 525–530, 2004.
25. Hallberg, B.M., Leitner, C., Haltrich, D., et al., Crystal structure of the 270 kDa homotetrameric lignin-degrading enzyme pyranose 2-oxidase, *J. Mol. Biol.*, 341(3), 781–796, 2004.
26. Bannwarth, M., Bastian, S., Heckmann-Pohl, D., et al., Crystal structure of pyranose 2-oxidase from the white-rot fungus *Peniophora* sp., *Biochemistry*, 43(37), 11683–11690, 2004.
27. Liu, T.E., Wolf, B., Geigert, J., et al., Convenient laboratory procedure for producing solid D-arabino-hexos-2-ulose (D-glucosone), *Carbohydrate Res.*, 113(1), 151–157, 1983.
28. Koths, K., Halenbeck, R., and Moreland, M., Synthesis of the antibiotic cortalcerone from D-glucose using pyranose 2-oxidase and a novel fungal enzyme, aldose-2-ulose dehydratase, *Carbohydrate Res.*, 232(1), 59–75, 1992.
29. Giffhorn, F., Fungal pyranose oxidases: occurrence, properties and biotechnical applications in carbohydrate chemistry, *Appl. Microbiol. Biotechnol.*, 54(6), 727–740, 2000.
30. Bastian, S., Rekowski, M.J., Witte, K., et al., Engineering of pyranose 2-oxidase from *Peniophora gigantea* towards improved thermostability and catalytic efficiency, *Appl. Microbiol. Biotechnol.*, 67(5), 654–663, 2005.
31. Xu, F., Golightly, E.J., Fuglsang, C.C., et al., A novel carbohydrate: acceptor oxidoreductase from *Microdochium nivale*, *Eur. J. Biochem.*, 268(4), 1136–1142, 2001.
32. Custers, J.H., Harrison, S.J., Sela-Buurlage, M.B., et al., Isolation and characterisation of a class of carbohydrate oxidases from higher plants, with a role in active defence, *Plant J.*, 39(2), 147–160, 2004.
33. Xu, F., Applications of oxidoreductases: recent progress, *Ind. Biotechnol.*, 1(1), 38–50, 2005.
34. Hiraga, K., Eto, T., Yoshioka, I., et al., Molecular cloning and expression of a gene encoding a novel sorbitol oxidase from *Streptomyces* sp. H-7775, *Biosci. Biotechnol. Biochem.*, 62(2), 347–353, 1998.
35. Yamashita, M., Omura, H., Okamoto, E., et al., Isolation, characterization, and molecular cloning of a thermostable xylitol oxidase from *Streptomyces* sp. IKD472, *J. Biosci. Bioeng.*, 89(4), 350–360, 2000.
36. Salusjarvi, T., Kalkkinen, N., and Miasnikov, A.N., Cloning and characterization of gluconolactone oxidase of *Penicillium cyaneo-fulvum* ATCC 10431 and evaluation of its use for production of D-erythorbic acid in recombinant *Pichia pastoris*, *Appl. Environ. Microbiol.*, 70(9), 5503–5510, 2004.
37. van der Klei, I.J., Harder, W., and Veenhuis, M., Biosynthesis and assembly of alcohol oxidase, a peroxisomal matrix protein in methylotrophic yeasts: a review, *Yeast*, 7(3), 195–209, 1991.
38. Vanhanen, S., West, M., Kroon, J.T., et al., A consensus sequence for long-chain fatty-acid alcohol oxidases from *Candida* identifies a family of genes involved in lipid ω -oxidation in yeast with homologues in plants and bacteria, *J. Biol. Chem.*, 275(6), 4445–4452, 2000.
39. Eirich, L.D., Craft, D.L., Steinberg, L., et al., Cloning and characterization of three fatty alcohol oxidase genes from *Candida tropicalis* strain ATCC 20336, *Appl. Environ. Microbiol.*, 70(8), 4872–4879, 2004.

40. Cheng, Q., Liu, H.T., Bombelli, P., et al., Functional identification of AtFao3, a membrane bound long chain alcohol oxidase in *Arabidopsis thaliana*, *FEBS Lett.*, 574(1–3), 62–68, 2004.
41. Yamashita, N., Motoyoshi, T., and Nishimura, A., Molecular cloning of the isoamyl alcohol oxidase-encoding gene (*mreA*) from *Aspergillus oryzae*, *J. Biosci. Bioeng.*, 89(3), 522–527, 2000.
42. Amar, D., North, P., Miskiniene, V., et al., Hydroxamates as substrates and inhibitors for FMN-dependent 2-hydroxy acid dehydrogenases, *Bioorg. Chem.*, 30(3), 145–162, 2002.
43. Yorita, K., Misaki, H., Palfey, B.A., et al., On the interpretation of quantitative structure–function activity relationship data for lactate oxidase, *Proc. Natl. Acad. Sci. USA*, 97(6), 2480–2485, 2000.
44. Kroutil, W., Mang, H., Edegger, K., et al., Biocatalytic oxidation of primary and secondary alcohols, *Adv. Synth. Catal.*, 346(2–3), 125–142, 2004.
45. Adam, W., Lazarus, M., Boss, B., et al., Enzymatic resolution of chiral 2-hydroxy carboxylic acids by enantioselective oxidation with molecular oxygen catalyzed by the glycolate oxidase from spinach (*Spinacia oleracea*), *J. Org. Chem.*, 62(22), 7841–7843, 1997.
46. Oikawa, T., Mukoyama, S., and Soda, K., Chemo-enzymatic D-enantiomerization of DL-lactate, *Biotechnol. Bioeng.*, 73(1), 80–82, 2001.
47. Leegsma-Vogt, G., Rhemrev-Boom, M.M., Tiessen, R.G., et al., The potential of biosensor technology in clinical monitoring and experimental research, *Biomed. Mater. Eng.*, 14(4), 455–464, 2004.
48. Poscia, A., Messeri, D., Moscone, D., et al., A novel continuous subcutaneous lactate monitoring system, *Biosens. Bioelectron.*, 20(11), 2244–2250, 2005.
49. Sampson, N.S. and Vrielink, A., Cholesterol oxidases: a study of nature's approach to protein design, *Acc. Chem. Res.*, 36(9), 713–722, 2003.
50. MacLachlan, J., Wotherspoon, A.T., Ansell, R.O., et al., Cholesterol oxidase: sources, physical properties and analytical applications, *J. Steroid Biochem. Mol. Biol.*, 72(5), 169–195, 2000.
51. Corbin, D.R., Grebenok, R.J., Ohnmeiss, T.E., et al., Expression and chloroplast targeting of cholesterol oxidase in transgenic tobacco plants, *Plant. Physiol.*, 126(3), 1116–1128, 2001.
52. Ahn, K.W. and Sampson, N.S., Cholesterol oxidase senses subtle changes in lipid bilayer structure, *Biochemistry*, 43(3), 827–836, 2004.
53. Sampson, N.S., Kass, I.J., and Ghoshroy, K.B., Assessment of the role of an omega loop of cholesterol oxidase: a truncated loop mutant has altered substrate specificity, *Biochemistry*, 37(16), 5770–5778, 1998.
54. Xiang, J. and Sampson, N.S., Library screening studies to investigate substrate specificity in the reaction catalyzed by cholesterol oxidase, *Protein Eng. Des. Sel.*, 17(4), 341–348, 2004.
55. van den Heuvel, R.H.H., Fraaije, M.W., Laane, C., et al., Regio- and stereospecific conversion of 4-alkylphenols by the covalent flavoprotein vanillyl-alcohol oxidase, *J. Bacteriol.*, 180(21), 5646–5651, 1998.
56. Mattevi, A., Fraaije, M.W., Mozzarelli, A., et al., Crystal structures and inhibitor binding in the octameric flavoenzyme vanillyl-alcohol oxidase: the shape of the active-site cavity controls substrate specificity, *Structure*, 5(7), 907–920, 1997.
57. Fraaije, M.W. and van Berkel, W.J.H., Catalytic mechanism of the oxidative demethylation of 4-(methoxymethyl)phenol by vanillyl-alcohol oxidase: evidence for formation of a *p*-quinone methide intermediate, *J. Biol. Chem.*, 272(29), 18111–18116, 1997.
58. Drijfhout, F.P., Fraaije, M.W., Jongejan, H., et al., Enantioselective hydroxylation of 4-alkylphenols by vanillyl alcohol oxidase, *Biotechnol. Bioeng.*, 59(2), 171–177, 1998.
59. van den Heuvel, R.H.H., Partridge, J., Laane, C., et al., Tuning of the product spectrum of vanillyl-alcohol oxidase by medium engineering, *FEBS Lett.*, 503(2–3), 213–216, 2001.
60. van den Heuvel, R.H.H., Fraaije, M.W., Ferrer, M., et al., Inversion of stereospecificity of vanillyl-alcohol oxidase, *Proc. Natl. Acad. Sci. USA*, 97(17), 9455–9460, 2000.
61. van den Heuvel, R.H., Fraaije, M.W., Laane, C., et al., Enzymatic synthesis of vanillin, *J. Agric. Food Chem.*, 49(6), 2954–2958, 2001.
62. van den Heuvel, R.H., van den Berg, W.A., Rovida, S., et al., Laboratory-evolved vanillyl-alcohol oxidase produces natural vanillin, *J. Biol. Chem.*, 279(32), 33492–33500, 2004.
63. Varela, E., Guillen, F., Martinez, A.T., et al., Expression of *Pleurotus eryngii* aryl-alcohol oxidase in *Aspergillus nidulans*: purification and characterization of the recombinant enzyme, *Biochim. Biophys. Acta.*, 1546(1), 107–113, 2001.

64. Ferreira, P., Medina, M., Guillen, F., et al., Spectral and catalytic properties of aryl-alcohol oxidase, a fungal flavoenzyme acting on polyunsaturated alcohols, *Biochem. J.*, 38g(3), 731–738, 2005.
65. Guillén, F., Martínez, Á.T., and Martínez, M.J., Substrate specificity and properties of the aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*, *Eur. J. Biochem.*, 209(2), 603–611, 1992.
66. Pilone, M.S. and Pollegioni, L., D-Amino acid oxidase as an industrial biocatalyst, *Biocat. Bio-transform.*, 20(3), 145–159, 2002.
67. Pollegioni, L., Caldinelli, L., Molla, G., et al., Catalytic properties of D-amino acid oxidase in cephalosporin C bioconversion: a comparison between proteins from different sources, *Biotechnol. Prog.*, 20(2), 467–473, 2004.
68. Tishkov, V.I. and Khoronenkova, S.V., D-Amino acid oxidase: structure, catalytic mechanism, and practical application, *Biochemistry* (Moscow), 70(1), 40–54, 2005.
69. Pilone, M.S., D-Amino acid oxidase: new findings, *Cell. Mol. Life Sci.*, 57(12), 1732–1747, 2000.
70. Betancor, L., Hidalgo, A., Fernandez-Lorente, G., et al., Use of physicochemical tools to determine the choice of optimal enzyme: stabilization of D-amino acid oxidase, *Biotechnol. Prog.*, 19(3), 784–788, 2003.
71. Ju, S.S., Lin, L.L., Chien, H.R., et al., Substitution of the critical methionine residues in *Trigonopsis variabilis* D-amino acid oxidase with leucine enhances its resistance to hydrogen peroxide, *FEMS Microbiol. Lett.*, 186(2), 215–219, 2000.
72. Pawelek, P.D., Cheah, J., Coulombe, R., et al., The structure of L-amino acid oxidase reveals the substrate trajectory into an enantiomerically conserved active site, *EMBO J.*, 19(16), 4204–4215, 2000.
73. Geyer, A., Fitzpatrick, T.B., Pawelek, P.D., et al., Structure and characterization of the glycan moiety of L-amino-acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*, *Eur. J. Biochem.*, 268(14), 4044–4053, 2001.
74. Geueke, B. and Hummel, W., Heterologous expression of *Rhodococcus opacus* L-amino acid oxidase in *Streptomyces lividans*, *Protein Expr. Purif.*, 28(2), 303–309, 2003.
75. Nishizawa, T., Aldrich, C.C. and Sherman, D.H., Molecular analysis of the rebeccamycin L-amino acid oxidase from *Lechevalieria aerocolonigenes* ATCC 39243, *J. Bacteriol.*, 187(6), 2084–2092, 2005.
76. Soda, K., Oikawa, T., and Yokoigawa, K., One-pot chemo-enzymatic enantiomerization of racemates, *J. Mol. Catal. B: Enzym.*, 11(4–6), 149–153, 2001.
77. Turner, N.J., Controlling chirality, *Curr. Opin. Biotechnol.*, 14(4), 401–406, 2003.
78. Enright, A., Alexandre, F.R., Roff, G., et al., Stereoinversion of beta- and gamma-substituted alpha-amino acids using a chemo-enzymatic oxidation–reduction procedure, *Chem. Commun.*, 2636–2637, 2003.
79. Binda, C., Newton-Vinson, P., Hubalek, F., et al., Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders, *Nat. Struct. Biol.*, 9(1), 22–26, 2002.
80. Roelofs, J. and Van Haastert, P.J., Genes lost during evolution, *Nature*, 411(6841), 1013–1014, 2001.
81. Setini, A., Pierucci, F., Senatori, O., et al., Molecular characterization of monoamine oxidase in zebrafish (*Danio rerio*), *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, 140(1), 153–161, 2005.
82. Schilling, B. and Lerch, K., Cloning, sequencing and heterologous expression of the monoamine oxidase gene from *Aspergillus niger*, *Mol. Gen. Genet.*, 247(4), 430–438, 1995.
83. Sablin, S.O., Yankovskaya, V., Bernard, S., et al., Isolation and characterization of an evolutionary precursor of human monoamine oxidases A and B, *Eur. J. Biochem.*, 253(1), 270–279, 1998.
84. Alexeeva, M., Carr, R., and Turner, N.J., Directed evolution of enzymes: new biocatalysts for asymmetric synthesis, *Org. Biomol. Chem.*, 1(23), 4133–4137, 2003.
85. Carr, R., Alexeeva, M., Dawson, M.J., et al., Directed evolution of an amine oxidase for the preparative deracemisation of cyclic secondary amines, *Chem. Biochem.*, 6(4), 637–639, 2005.
86. Newton-Vinson, P., Hubalek, F., and Edmondson, D.E., High-level expression of human liver monoamine oxidase B in *Pichia pastoris*, *Protein Express. Purif.*, 20(2), 334–345, 2000.
87. Li, M., Hubalek, F., Newton-Vinson, P., et al., High-level expression of human liver monoamine oxidase A in *Pichia pastoris*: comparison with the enzyme expressed in *Saccharomyces cerevisiae*, *Protein Express. Purif.*, 24(1), 152–162, 2002.

88. Edmondson, D.E., Binda, C., and Mattevi, A., The FAD binding sites of human monoamine oxidases A and B, *Neurotoxicology*, 25(1–2), 63–72, 2004.
89. Binda, C., Li, M., Hubalek, F., et al., Insights into the mode of inhibition of human mitochondrial monoamine oxidase B from high-resolution crystal structures, *Proc. Natl. Acad. Sci. USA*, 100(17), 9750–9755, 2003.
90. Hubalek, F., Binda, C., Khalil, A., et al., Demonstration of isoleucine 199 as a structural determinant for the selective inhibition of human monoamine oxidase B by specific reversible inhibitors, *J. Biol. Chem.*, 280(16), 15761–15766, 2005.
91. Scrutton, N.S., Chemical aspects of amine oxidation by flavoprotein enzymes, *Nat. Prod. Rep.*, 21(6), 722–730, 2004.
92. Edmondson, D.E., Mattevi, A., Binda, C., et al., Structure and mechanism of monoamine oxidase, *Curr. Med. Chem.*, 11(15), 1983–1993, 2004.
93. Rigby, S.E., Hynson, R.M., Ramsay, R.R., et al., A stable tyrosyl radical in monoamine oxidase A, *J. Biol. Chem.*, 280(6), 4627–4631, 2005.
94. Markoglou, N., Hsuesh, R., and Wainer, I.W., Immobilized enzyme reactors based upon the flavoenzymes monoamine oxidase A and B, *J. Chromatogr. B*, 804(2), 295–302, 2004.
95. Chen, G., Yee, D.J., Gubernator, N.G., et al., Design of optical switches as metabolic indicators: new fluorogenic probes for monoamine oxidases (MAO A and B), *J. Am. Chem. Soc.*, 127(13), 4544–4545, 2005.
96. Yoshida, N., Sakai, Y., Isogai, A., et al., Primary structures of fungal fructosyl amino acid oxidases and their application to the measurement of glycated proteins, *Eur. J. Biochem.*, 242(3), 499–505, 1996.
97. Akazawa, S., Karino, T., Yoshida, N., et al., Functional analysis of fructosyl-amino acid oxidases of *Aspergillus oryzae*, *Appl. Environ. Microbiol.*, 70(10), 5882–5890, 2004.
98. Yoshida, N., Takatsuka, K., Katsuragi, T., et al., Occurrence of fructosyl-amino acid oxidase-reactive compounds in fungal cells, *Biosci. Biotechnol. Biochem.*, 69(1), 258–260, 2005.
99. Sakaue, R. and Kajiyama, N., Thermostabilization of bacterial fructosyl-amino acid oxidase by directed evolution, *Appl. Environ. Microbiol.*, 69(1), 139–145, 2003.
100. Thorpe, C., Hooper, K.L., Raje, S., et al., Sulfhydryl oxidases: emerging catalysts of protein disulfide bond formation in eukaryotes, *Arch. Biochem. Biophys.*, 405(1), 1–12, 2002.
101. Gross, E., Kastner, D.B., Kaiser, C.A., et al., Structure of Ero1p, source of disulfide bonds for oxidative protein folding in the cell, *Cell*, 117(5), 601–610, 2004.
102. Vignaud, C., Kaid, N., Rakotozafy, S., et al., Partial purification and characterization of sulfhydryl oxidase from *Aspergillus niger*, *J. Food Sci.*, 67, 2016–2022, 2002.
103. Vemulapulli, V., Miller, R., and Hosney, R.C., Glucose oxidase in breadmaking systems, *Cereal Chem.*, 75(2), 439–442, 1998.
104. Poulsen, C. and Bak Hostrup, P., Purification and characterization of a hexose oxidase with excellent strengthening effects in bread, *Cereal Chem.*, 75(1), 51–57, 1998.
105. Wagner, M.A., Trickey, P., Chen, Z.W., et al., Monomeric sarcosine oxidase, 1: flavin reactivity and active site binding determinants, *Biochemistry*, 39(30), 8813–8824, 2000.
106. Binda, C., Coda, A., Angelini, R., et al., A 30-angstrom-long U-shaped catalytic tunnel in the crystal structure of polyamine oxidase, *Structure Fold. Des.*, 7(3), 265–276, 1999.
107. Huang, Q., Liu, Q., and Hao, Q., Crystal structures of Fms1 and its complex with spermine reveal substrate specificity, *J. Mol. Biol.*, 348(4), 951–959, 2005.
108. Fitzpatrick, P.F., Orville, A.M., Nagpal, A., et al., Nitroalkane oxidase, a carbanion-forming flavoprotein homologous to acyl-CoA dehydrogenase, *Arch. Biochem. Biophys.*, 433(1), 157–165, 2005.
109. Valley, M.P., Tichy, S.E., and Fitzpatrick, P.F., Establishing the kinetic competency of the cationic imine intermediate in nitroalkane oxidase, *J. Am. Chem. Soc.*, 127(7), 2062–2066, 2005.
110. Chiribau, C.B., Sandu, C., Fraaije, M., et al., A novel gamma-N-methylaminobutyrate demethylating oxidase involved in catabolism of the tobacco alkaloid nicotine by *Arthrobacter nicotinovorans* pAO1, *Eur. J. Biochem.*, 271(23–24), 4677–4684, 2004.
111. Entsch, B. and van Berkel, W.J., Structure and mechanism of *para*-hydroxybenzoate hydroxylase, *FASEB J.*, 9(7), 476–483, 1995.

112. Enroth, C., Neujahr, H., Schneider, G., et al., The crystal structure of phenol hydroxylase in complex with FAD and phenol provides evidence for a concerted conformational change in the enzyme and its cofactor during catalysis, *Structure*, 6(5), 605–617, 1998.
113. Malito, E., Alfieri, A., Fraaije, M.W., et al., Crystal structure of a Baeyer–Villiger monooxygenase, *Proc. Natl. Acad. Sci. USA*, 101(36), 13157–13162, 2004.
114. Schmid, A., Dordick, J.S., Hauer, B., et al., Industrial biocatalysis today and tomorrow, *Nature*, 409(6817), 258–268, 2001.
115. Wierenga, R.K., de Jong, R.J., Kalk, K.H., et al., Crystal structure of *p*-hydroxybenzoate hydroxylase, *J. Mol. Biol.*, 131(1), 55–73, 1979.
116. Schreuder, H.A., Prick, P.A., Wierenga, R.K., et al., Crystal structure of the *p*-hydroxybenzoate hydroxylase–substrate complex refined at 1.9 Å resolution: analysis of the enzyme–substrate and enzyme–product complexes, *J. Mol. Biol.*, 208(4), 679–696, 1989.
117. Entsch, B., Cole, L.J., and Ballou, D.P., Protein dynamics and electrostatics in the function of *p*-hydroxybenzoate hydroxylase, *Arch. Biochem. Biophys.*, 433(1), 297–311, 2005.
118. Mattevi, A., The PHBH fold: not only flavoenzymes, *Biophys. Chem.*, 70(3), 217–222, 1998.
119. Moonen, M.J.H., Fraaije, M.W., Rietjens, I.M.C.M., et al., Flavoenzyme-catalyzed oxygenations and oxidations of phenolic compounds, *Adv. Synth. Catal.*, 344(10), 1023–1035, 2002.
120. Peelen, S., Rietjens, I.M.C.M., Boersma, M.G., et al., Conversion of phenol derivatives to hydroxylated products by phenol hydroxylase from *T. cutaneum*: a comparison of regioselectivity and rate of conversion with calculated molecular orbital substrate characteristics, *Eur. J. Biochem.*, 227(1–2), 284–291, 1995.
121. van der Bolt, F.J.T., van den Heuvel, R.H.H., Vervoort, J., et al., ¹⁹F NMR study on the regiospecificity of hydroxylation of tetrafluoro-4-hydroxybenzoate by wild-type and Y385F *p*-hydroxybenzoate hydroxylase: evidence for a consecutive oxygenolytic dehalogenation mechanism, *Biochemistry*, 36(46), 14192–14201, 1997.
122. Jadan, A.P., Moonen, M.J.H., Boeren, S.A., et al., Biocatalytic potential of *p*-hydroxybenzoate hydroxylase from *Rhodococcus rhodnii* 135 and *Rhodococcus opacus* 557, *Adv. Synth. Catal.*, 346(2–3), 367–375, 2004.
123. Kälén, M., Neujahr, H.Y., Weissmahr, R.N., et al., Phenol hydroxylase from *Trichosporon cutaneum*: gene cloning, sequence analysis, and functional expression in *Escherichia coli*, *J. Bacteriol.*, 174(22), 7112–7120, 1992.
124. Held, M., Suske, W., Schmid, A., et al., Preparative scale production of 3-substituted catechols using a novel monooxygenase from *Pseudomonas azelaica* HBP 1, *J. Mol. Catal. B: Enzym.*, 5(1–4), 87–93, 1998.
125. Held, M., Schmid, A., Kohler, H.P., et al., An integrated process for the production of toxic catechols from toxic phenols based on a designer biocatalyst, *Biotechnol. Bioeng.*, 62(6), 641–648, 1999.
126. Schmid, A., Vereyken, I., Held, M., et al., Preparative regio- and chemoselective functionalization of hydrocarbons catalyzed by cell free preparations of 2-hydroxybiphenyl 3-monooxygenase, *J. Mol. Catal. B: Enzym.*, 11(4–6), 455–462, 2001.
127. Meyer, A., Schmid, A., Held, M., et al., Changing the substrate reactivity of 2-hydroxybiphenyl 3-monooxygenase from *Pseudomonas azelaica* HBP1 by directed evolution, *J. Biol. Chem.*, 277(7), 5575–5582, 2002.
128. Meyer, A., Wursten, M., Schmid, A., et al., Hydroxylation of indole by laboratory evolved 2-hydroxybiphenyl 3-monooxygenase, *J. Biol. Chem.*, 277(37), 34161–34167, 2002.
129. Meyer, A., Held, M., Schmid, A., et al., Synthesis of 3-*tert*-butylcatechol by an engineered monooxygenase, *Biotechnol. Bioeng.*, 81(5), 518–524, 2003.
130. Kamerbeek, N.M., Janssen, D.B., van Berkel, W.J.H., et al., Baeyer–Villiger monooxygenases, an emerging family of flavin-dependent biocatalysts, *Adv. Synth. Catal.*, 345(6–7), 667–678, 2003.
131. Fraaije, M.W., Kamerbeek, N.M., van Berkel, W.J., et al., Identification of a Baeyer–Villiger monooxygenase sequence motif, *FEBS Lett.*, 518(1–3), 43–47, 2002.
132. Cashman, J.R., The implications of polymorphisms in mammalian flavin-containing monooxygenases in drug discovery and development, *Drug Discov. Today*, 9(13), 574–581, 2004.
133. Walsh, C.T. and Chen, Y.-C.J., Enzymatic Baeyer–Villiger-oxidations by flavin-dependent monooxygenases, *Angew. Chem. Int. Ed. Engl.*, 100(2), 342–352, 1988.

134. Mihovilovic, M.D., Müller, B., and Stanetty, P., Monooxygenase-mediated Baeyer–Villiger oxidations, *Eur. J. Org. Chem.*, 2002(22), 3711–3730, 2002.
135. Alphand, V., Carrea, G., Wohlgemuth, R., et al., Towards large-scale synthetic applications of Baeyer–Villiger monooxygenases, *Trends Biotechnol.*, 21(7), 318–323, 2003.
136. Ottolina, G., de Gonzalo, G., Carrea, G., et al., Enzymatic Baeyer–Villiger oxidation of bicyclic diketones, *Adv. Synth. Catal.*, 347(7–8), 1035–1040, 2005.
137. Gutierrez, M.-C., Furstoss, R., and Alphand, V., Microbial transformations 60: enantioconvergent Baeyer–Villiger oxidation via a combined whole cells and ionic exchange resin-catalysed dynamic resolution process, *Adv. Synth. Catal.*, 347(7–8), 1051–1059, 2005.
138. Fraaije, M.W., Wu, J., Heuts, D.P., et al., Discovery of a thermostable Baeyer–Villiger monooxygenase by genome mining, *Appl. Microbiol. Biotechnol.*, 66(4), 393–400, 2005.
139. Moonen, M.J.H., Westphal, A.H., Rietjens, I.M.C.M., et al., Enzymatic Baeyer–Villiger oxidation of benzaldehydes, *Adv. Synth. Catal.*, 347(7–8), 1027–1034, 2005.
140. Mihovilovic, M.D., Kapitan, P., Rydz, J., et al., Biooxidation of ketones with a cyclobutanone structural motif by recombinant whole-cells expressing 4-hydroxyacetophenone monooxygenase, *J. Mol. Catal. B Enzym.*, 32(1), 135–140, 2005.
141. Sicard, R., Chen, L.S., Marsaioli, A.J., et al., A fluorescence-based assay for Baeyer–Villiger monooxygenases, hydroxylases and lactonases, *Adv. Synth. Catal.*, 347(7–8), 1041–1050, 2005.
142. Sheng, D., Ballou, D.P., and Massey, V., Mechanistic studies of cyclohexanone monooxygenase: chemical properties of intermediates involved in catalysis, *Biochemistry*, 40(37), 11156–11167, 2001.
143. Bocola, M., Schulz, F., Leca, F., et al., Converting phenylacetone monooxygenase into phenylcyclohexanone monooxygenase by rational design: towards practical Baeyer–Villiger monooxygenases, *Adv. Synth. Catal.*, 347(7–8), 979–986, 2005.
144. Fraaije, M.W., Kamerbeek, N.M., Heidekamp, A.J., et al., The prodrug activator EtaA from *Mycobacterium tuberculosis* is a Baeyer–Villiger monooxygenase, *J. Biol. Chem.*, 279(5), 3354–3360, 2004.
145. Kirchner, U., Westphal, A.H., Muller, R., et al., Phenol hydroxylase from *Bacillus thermoglucosidasius* A7, a two-protein component monooxygenase with a dual role for FAD, *J. Biol. Chem.*, 278(48), 47545–47553, 2003.
146. Thotsaporn, K., Sucharitakul, J., Wongratana, J., et al., Cloning and expression of *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii*: evidence of the divergence of enzymes in the class of two-protein component aromatic hydroxylases, *Biochim. Biophys. Acta*, 1680(1), 60–66, 2004.
147. van den Heuvel, R.H., Westphal, A.H., Heck, A.J., et al., Structural studies on flavin reductase PheA2 reveal binding of NAD in an unusual folded conformation and support novel mechanism of action, *J. Biol. Chem.*, 279(13), 12860–12867, 2004.
148. Prieto, M.A. and Garcia, J.L., Molecular characterization of 4-hydroxyphenylacetate 3-hydroxylase of *Escherichia coli*: a two-protein component enzyme, *J. Biol. Chem.*, 269(36), 22823–22829, 1994.
149. Hartmans, S., van der Werf, M.J., and de Bont, J.A., Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase, *Appl. Environ. Microbiol.*, 56(5), 1347–1351, 1990.
150. Panke, S., Witholt, B., Schmid, A., et al., Towards a biocatalyst for (*S*)-styrene oxide production: characterization of the styrene degradation pathway of *Pseudomonas* sp. strain VLB120, *Appl. Environ. Microbiol.*, 64(6), 2032–2043, 1998.
151. Di Gennaro, P., Colmegna, A., Galli, E., et al., A new biocatalyst for production of optically pure aryl epoxides by styrene monooxygenase from *Pseudomonas fluorescens* ST, *Appl. Environ. Microbiol.*, 65(6), 2794–2797, 1999.
152. O’Leary, N.D., Duetz, W.A., Dobson, A.D., et al., Induction and repression of the sty operon in *Pseudomonas putida* CA-3 during growth on phenylacetic acid under organic and inorganic nutrient-limiting continuous culture conditions, *FEMS Microbiol. Lett.*, 208(2), 263–268, 2002.
153. Otto, K., Hofstetter, K., Rothlisberger, M., et al., Biochemical characterization of StyAB from *Pseudomonas* sp. strain VLB120 as a two-component flavin-diffusible monooxygenase, *J. Bacteriol.*, 186(16), 5292–5302, 2004.

154. Hollmann, F., Hofstetter, K., Habicher, T., et al., Direct electrochemical regeneration of mono-oxygenase subunits for biocatalytic asymmetric epoxidation, *J. Am. Chem. Soc.*, 127(18), 6540–6541, 2005.
155. Gray, K.A., Mrachko, G.T., and Squires, C.H., Biodesulfurization of fossil fuels, *Curr. Opin. Microbiol.*, 6(3), 229–235, 2003.
156. Denome, S.A., Oldfield, C., Nash, L.J., et al., Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8, *J. Bacteriol.*, 176(21), 6707–6716, 1994.
157. Piddington, C.S., Kovacevich, B.R., and Rambosek, J., Sequence and molecular characterization of a DNA region encoding the dibenzothiophene desulfurization operon of *Rhodococcus* sp. strain IGTS8, *Appl. Environ. Microbiol.*, 61(2), 468–475, 1995.
158. Matsubara, T., Ohshiro, T., Nishina, Y., et al., Purification, characterization, and overexpression of flavin reductase involved in dibenzothiophene desulfurization by *Rhodococcus erythropolis* D-1, *Appl. Environ. Microbiol.*, 67(3), 1179–1184, 2001.
159. Galan, B., Diaz, E., and Garcia, J.L., Enhancing desulphurization by engineering a flavin reductase-encoding gene cassette in recombinant biocatalysts, *Environ. Microbiol.*, 2(6), 687–694, 2000.
160. Reichmuth, D.S., Hittle, J.L., Blanch, H.W., et al., Biodesulfurization of dibenzothiophene in *Escherichia coli* is enhanced by expression of a *Vibrio harveyi* oxidoreductase gene, *Biotechnol. Bioeng.*, 67(1), 72–79, 2000.
161. Arensdorf, J.J., Loomis, A.K., DiGrazia, P.M., et al., Chemostat approach for the directed evolution of biodesulfurization gain-of-function mutants, *Appl. Environ. Microbiol.*, 68(2), 691–698, 2002.
162. Coco, W.M., Levinson, W.E., Crist, M.J., et al., DNA shuffling method for generating highly recombined genes and evolved enzymes, *Nat. Biotechnol.*, 19(4), 354–359, 2001.
163. Keller, S., Wage, T., Hohaus, K., et al., Purification and partial characterization of tryptophan 7-halogenase (PrnA) from *Pseudomonas fluorescens*, *Angew. Chem. Int. Ed. Engl.*, 39(13), 2300–2302, 2000.
164. Burd, V.N. and van Pee, K.H., Cloning and sequencing of the gene of tryptophan-7-halogenase from *Pseudomonas fluorescens* strain CHA0, *Biochemistry (Moscow)*, 69(6), 674–677, 2004.
165. Yeh, E., Garneau, S., and Walsh, C.T., Robust *in vitro* activity of RebF and RebH, a two-component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis, *Proc. Natl. Acad. Sci. USA*, 102(11), 3960–3965, 2005.
166. van Pee, K.H. and Unversucht, S., Biological dehalogenation and halogenation reactions, *Chemosphere*, 52(2), 299–312, 2003.
167. Unversucht, S., Hollmann, F., Schmid, A., et al., FADH₂-dependence of tryptophan 7-halogenase, *Adv. Synth. Catal.*, 347(7–8), 1163–1167, 2005.
168. Dong, C., Kotzsch, A., Dorward, M., et al., Crystallization and X-ray diffraction of a halogenating enzyme, tryptophan 7-halogenase, from *Pseudomonas fluorescens*, *Acta Crystall. Section D*, 60(8), 1438–1440, 2004.

7 Preparation of Chiral Pharmaceuticals through Enzymatic Acylation of Alcohols and Amines

Vicente Gotor-Fernández, Francisca Rebolledo, and Vicente Gotor

CONTENTS

7.1	Introduction	204
7.2	Enzymatic Acylation of Alcohols	206
7.2.1	Resolution of Monoalcohols	207
7.2.1.1	Aminoglutethimide (1)	207
7.2.1.2	Clavularin A and Clavularin B (7 and 8)	207
7.2.1.3	Coronafacic Acid (11)	207
7.2.1.4	(+)-Docosa-4,15-dien-1-yn-3-ol (14)	209
7.2.1.5	E-4018 (16)	209
7.2.1.6	Epothilone A (19)	210
7.2.1.7	Fluoxetine (22), Tomoxetine (23), and Nisoxetine (24)	210
7.2.1.8	Formoterol (30)	210
7.2.1.9	HMG CoA Inhibitors	211
7.2.1.10	β -Lactams	211
7.2.1.11	3-Methylnonacosanol (38)	212
7.2.1.12	Nifenalol (41) and Sotalol (42)	213
7.2.1.13	Nilvadipine (45)	213
7.2.1.14	OPC-29030 (48)	214
7.2.1.15	Propanolol (51)	215
7.2.1.16	Serotonin Antagonism and Uptake Inhibitors	215
7.2.2	Resolution of Diols	217
7.2.2.1	Baclofen (71)	217
7.2.2.2	Camptosar (74)	217
7.2.2.3	Cispentacin (77)	217
7.2.2.4	ϵ -Hydroxytramadol (81) and δ -Hydroxytramadol (82)	218
7.2.2.5	Leustroductin B (86)	220
7.2.2.6	Propanolol (51)	220
7.2.2.7	SCH 51048 (91)	220
7.2.2.8	Sobrerol (94)	221

7.2.2.9	Spyrotryprostatins A (96) and B (97), (–)-Physostigmine (98), and (–)-Esermethole (99)	221
7.2.3	Resolution of Polyalcohols	222
7.2.3.1	Glycerol Derivatives	222
7.2.3.2	Inositol Phosphates	222
7.2.3.3	Phosphonotrixin (114)	224
7.2.3.4	(<i>S</i>)- α -Tocotrienol (118)	224
7.3	Enzymatic Acylation of Aminoalcohols	225
7.3.1	(5 <i>S</i> ,9 <i>S</i>)-(+)-Indolizidine 209D (122)	225
7.3.2	Jatropham (125)	225
7.3.3	Norphenylephrine (127) and Octopamine (128)	226
7.4	Enzymatic Acylation of Amines	226
7.4.1	Resolution of Amines	226
7.4.1.1	Cinacalcet (133)	226
7.4.1.2	(<i>R</i>)- <i>N</i> -Propargyl-1-Aminoindan: Rasagiline (138)	227
7.4.1.3	1-[(2-Benzofuranyl)phenylmethyl]imidazols 142	229
7.4.1.4	1-Arylpropan-2-Amines (146): Amphetamine and Derivatives	229
7.4.1.5	Mexiletine (151)	230
7.4.1.6	Labetalol (153)	231
7.4.1.7	YH1885 (157)	232
7.4.1.8	SCH66336 (161)	232
7.4.2	Resolution of 1,2-Diamines	234
7.4.2.1	<i>trans</i> -Cyclohexane-1,2-Diamine Derivatives: Oxaliplatin (167) and NDDP (168)	234
7.4.2.2	U-50,488 (172)	234
7.5	Resolution of Esters by Aminolysis or Ammonolysis	235
7.5.1	Ibuprofen	235
7.5.2	3-Pyrrolidinol Derivatives 178–181	236
7.5.3	(<i>R</i>)-GABOB (185)	236
7.5.4	Roxifiban (189)	238
7.6	Resolution of Amino Acid Derivatives by <i>N</i> -Acylation Reactions	238
7.6.1	Loracarbef (193)	238
7.6.2	Proline and Pipelicolic Acid Derivatives 199–202	239
7.6.3	Moiramide B (208) and Andrimid (209)	240
7.6.4	Xemilofiban (212) and FR-184764 (213)	241
7.6.5	Amipurimycin (217)	242
7.7	Concluding Remarks	242
	References	243

7.1 INTRODUCTION

Nowadays, biocatalysis is a standard methodology for the production of chemicals. Biocatalytic steps are already in use to produce a wide range of products, including agricultural chemicals, drugs, and important commodity chemicals such as acrylamide [1]. These biocatalytic reactions can be carried out in organic solvents or in aqueous media. However, biocatalysis in nonconventional media has shown to be an excellent strategy for organic chemists in the production of chemicals that are difficult to obtain by chemical conventional procedures [2].

The use of enzymes in organic solvents is currently of special relevance for the preparation of products of fine chemicals. Recently, tremendous efforts have been made to establish enantioselective routes for the preparation of enantiomerically pure compounds due to their importance in the pharmaceutical, agricultural, and food industries [3]. Among the enzymes tested, lipases are the biocatalysts that have shown the greatest utility, especially through enzymatic transesterification reactions. In the last few years, there has been an ever-increasing trend for chiral drug substances to focus on single enantiomers instead of racemic mixtures. The most important reason for developing stereochemically pure and defined compounds is the difference of biological activity, displayed in many cases by each enantiomer of a chiral compound [4].

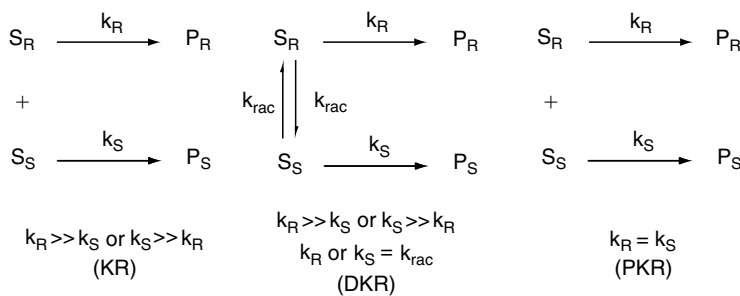
In this context, biocatalysis has been well recognized as an excellent strategy for the preparation of chiral pharmaceuticals [5]. Biocatalytic processes are environment-friendly in contrast to conventional chemical catalysis, especially when these make use of heavy metal catalysis. The role of biotransformations in the pharmaceutical, fine chemicals, or food industries is clearly expanding, as the pharmaceutical companies largely use this methodology [6]. In addition, the application of enzyme technology in the chemical industry has recently been well documented [7].

Both isolated enzymes and whole cells, either in soluble or immobilized form, have been used successfully in the synthesis of pharmacologically valuable materials [8,9]. However, hydrolytic enzymes, especially lipases, are widely used in organic synthesis as environment-friendly catalysts that possess broad substrate specificities, display high stereoselectivity, are commercially available, and do not require the use of cofactors [10].

These biocatalysts have been exploited for asymmetric synthesis transformations, led by the growing demand for enantiopure pharmaceuticals. Furthermore, lipase-catalyzed reactions are normally carried out under mild conditions, and can be used in organic solvents. In addition, biocatalysis in nonaqueous media has been widely used for the resolution of alcohols, acids, or lactones through enzymatic transesterification reactions using different lipases [11]. Moreover, other processes, such as the enzymatic acylation of amines or ammonia, have shown themselves to be of great utility for the resolution of amines and the preparation of chiral amides [12].

The main difference between enzymatic acylation of alcohols and amines is the use of the corresponding acyl donor, because activated esters that are of utility in acylation of alcohols react with amines in the absence of a biocatalyst, and nonactivated esters must be used to carry out an enzymatic aminolysis or ammonolysis reaction. In addition, lipases are the most efficient hydrolases to catalyze the acylation of amines and ammonia, because these hydrolytic enzymes have very low amidase activity, although in some cases the hydrolysis or alcoholysis of amides can be useful to achieve chiral amines [13].

Stereoselective biotransformations can be grouped into two different classes: asymmetric synthesis and kinetic resolution (KR) of racemic mixtures. Conceptually, they differ from each other in the fact that the asymmetric synthesis implies the formation of one or more chirality elements in a substrate, a KR is based on a transformation which, subsequently, makes easier the separation of the two enantiomers of the racemic substrate. This fact involves a practical difference: in a KR only half of the starting material is used (Scheme 7.1). When only one enantiomer of a substrate is required this constitutes a disadvantage of KRs and different approaches have been developed to overcome this limitation [14]. More attention has been recently paid in the dynamic kinetic resolution (DKR) [15] and consists in carrying out an *in situ* continuous racemization of the substrate, so that, theoretically, all of the racemic starting material can be used for transformation into one enantiomer. This new strategy has appeared in asymmetric catalysis during the last decade and the most common methodology involves a lipase as biocatalyst and a metal-organic complex as chemical catalyst [16]. Although, DKR of alcohols has been widely used for their resolution by enzymatic hydrolysis or transesterification, the application to the resolution of amines has been much less investigated [17]. A new



SCHEME 7.1 Kinetic resolution (KR), dynamic kinetic resolution (DKR), and parallel kinetic resolution (PKR).

concept has been demonstrated in the resolution of racemic mixtures in which both enantiomers of a substrate react with similar rate to give two different products with high enantiomeric excess, this strategy was called by Vedejs and Chen as parallel kinetic resolution (PKR) [18]. This concept in stereoselective biocatalysis has been recently reviewed [19].

As mentioned above there is other possibility, of great interest is the desymmetrization of proquiral or *meso* compounds using hydrolytic enzymes. The desymmetrization of symmetric compounds consists of a modification that eliminates one or more elements of symmetry of the substrate. If the symmetry elements that preclude chirality are eliminated, enantioselectivity can be achieved [20]. Enantioselective enzymatic desymmetrizations (EEDs) belong to the field of asymmetric synthesis and, accordingly, a maximum yield of 100% can be attained [21]. For this reason, they constitute a very interesting alternative to KRs for the preparation of optically active compounds, which is reflected in the increasing number of enzymatic desymmetrizations applied to synthesis published in the literature during the recent years [22].

To date, the enzymatic acylation of alcohols in organic solvents catalyzed by lipases through KR is still one of the most popular methods for the resolution of different hydroxyl compounds, although other types of resolutions such as DKR are increasing with time. Among the different acyl donors used in these resolutions, the most efficient are vinyl esters [23]. These esters can also be employed in EEDs for the desymmetrization of proquiral or *meso*-diol compounds by an enzymatic acylation of alcohols in organic solvents. Depending on the substrate, enzymatic resolution and desymmetrization processes have shown their utility in the preparation of pharmaceutical intermediates [24].

This chapter covers a wide range of resolutions of racemates and desymmetrization of proquiral or *meso* compounds through enzymatic acylation of alcohols and amines, using lipases for the preparation of a number of pharmaceuticals and chiral building blocks in the synthesis of chiral drugs that has been described in the literature. The enzymatic resolution of pharmaceuticals through the acylation of the hydroxyl group or hydrolysis of esters has also been reviewed in the last decade for many authors [1,5,6,8,16,22–34]. The enantioselective processes have been considered without including examples where biocatalyst has been used for the regioselective modification of different derivatives.

In this chapter, we have classified the enzymatic processes depending on the starting material in the reaction: alcohol, aminoalcohol, and amine derivatives.

7.2 ENZYMATIC ACYLATION OF ALCOHOLS

Among the alcohol derivatives, we have divided the section depending on the number of hydroxyl groups present in the molecule, in this manner we have three different divisions: monoalcohol, diol, and polyalcohol derivatives.

7.2.1 RESOLUTION OF MONOALCOHOLS

7.2.1.1 Aminogluthethimide (1)

It was originally developed as an anticonvulsant and now this itself and other derivatives are effective drugs against breast cancer for postmenopausal patients. The enzymatic transesterification of racemic 2-ethyl-5-hydroxy-2-phenylpentamonitrile (**2**) was done with different lipases (Scheme 7.2) obtaining the best results with *Pseudomonas cepacia* lipase (PSL) and *Pseudomonas fluorescens* lipase (LAK) [35]. Enzymatic acylation of **2** by PSL in hexane provided (*R*)-alcohol with 99% ee at 66% conversion after 8 h, while using LAK in diisopropyl ether (DIPE) (*S*)-alcohol is obtained with 96% ee at 86% conversion after 6 h.

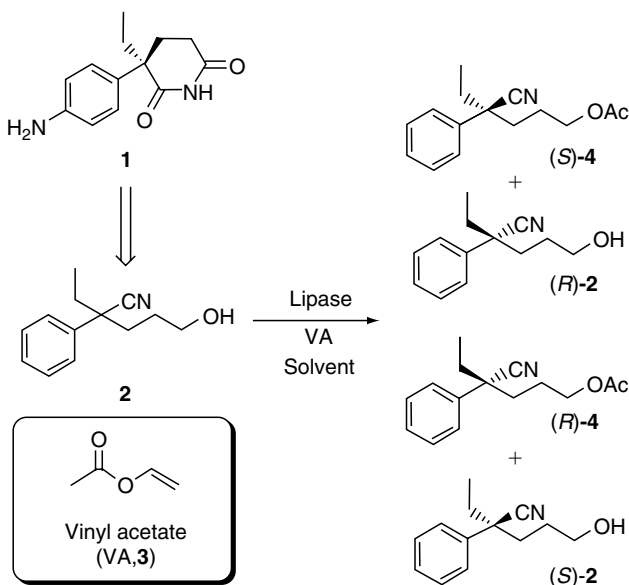
The cholesterol derivative **5** is a cholesterol metabolite that has been used as the (25*RS*)-epimeric mixture in studies concerning the inhibitory effects of oxysterols on the hydroxymethylglutarate coenzyme (HMG CoA) reductase activity. The 3 β -*O*-silyl ether of (25*RS*)-**5** was subjected to an acylation process with vinyl acetate catalyzed by PSL in chloroform affording the (25*R*)-acetate **6** in 30% conversion after 24 h (Scheme 7.3) [36].

7.2.1.2 Clavularin A and Clavularin B (7 and 8)

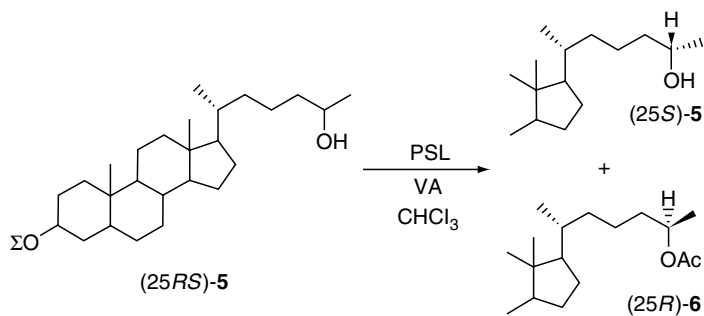
These anticancer agents were synthesized from (\pm)-**9** by transesterification reaction catalyzed by lipase LIP (from *Pseudomonas* sp.) using vinyl acetate in *tert*-butyl methyl ether (TBME) [37]. After 12 d at room temperature, (–)-acetate **10** was obtained in 36% yield and 95% ee, while (+)-alcohol **9** was recovered in 43% yield and 55% ee (Scheme 7.4).

7.2.1.3 Coronafacic Acid (11)

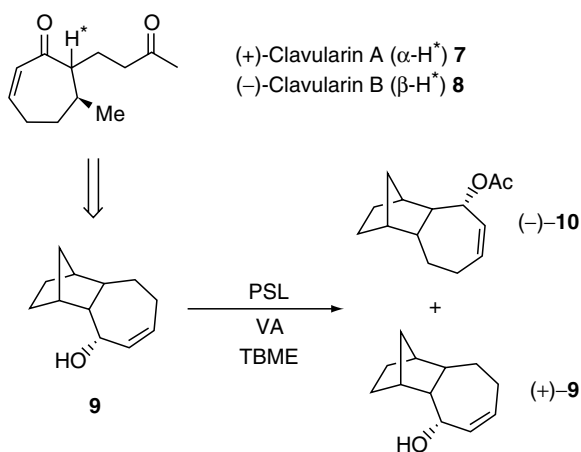
Compound (+)-**11** is a common chiral building block of natural products present in diverse pharmaceuticals. The resolution of racemic **12** in THF catalyzed by PSL supported on celite using vinyl acetate as acyl donor at room temperature for 4 h (Scheme 7.5) afforded allylic acetate (–)-**13** (>98% ee and 44% yield) and (+)-alcohol **12** (>99% ee and 46% yield) [38].



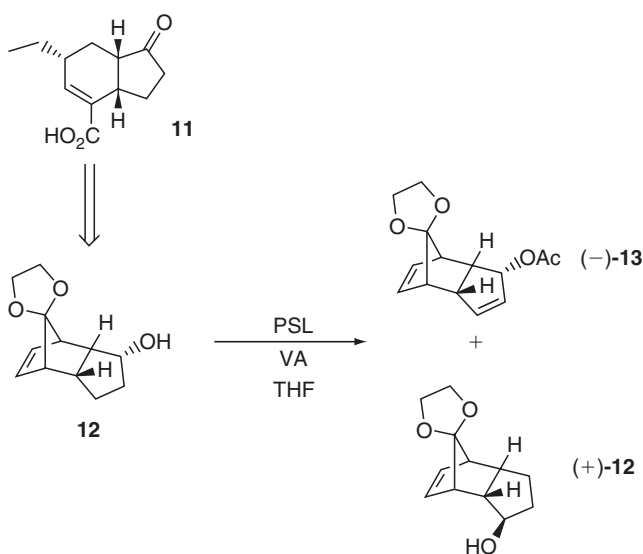
SCHEME 7.2 Enzymatic resolution of precursor **2** for the synthesis of (*R*)-aminogluthethimide **1**.



SCHEME 7.3 Enzymatic resolution of **5**.



SCHEME 7.4 Enzymatic resolution of precursor **9** for the synthesis of clavularin A and clavularin B.



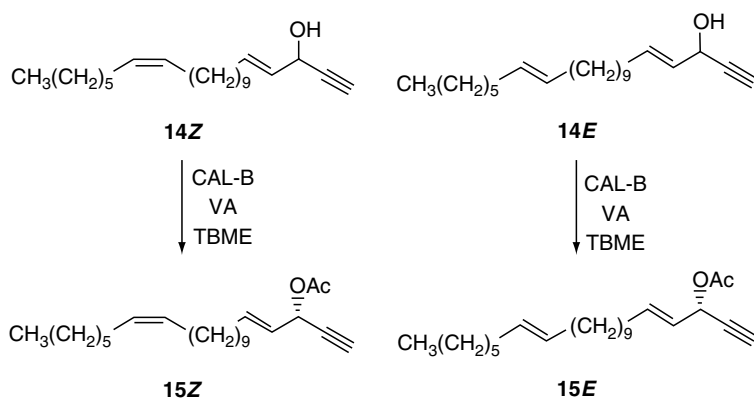
SCHEME 7.5 Enzymatic resolution of precursor **12** for the synthesis of coronafacic acid.

7.2.1.4 (+)-Docosa-4,15-dien-1-yn-3-ol (**14**)

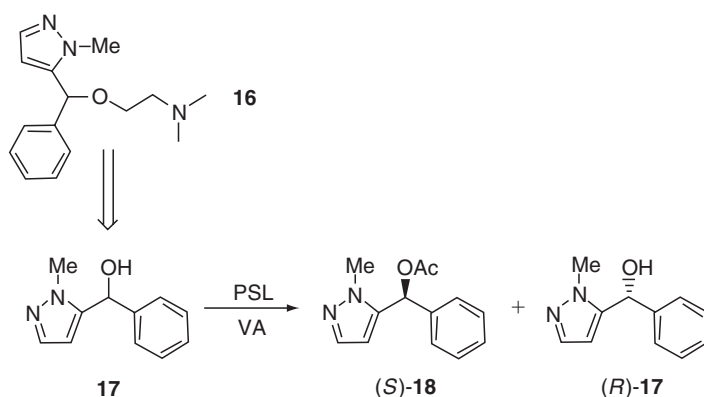
Compound **22** shows *in vitro* immunosuppressive and antitumor activities. The lipase-catalyzed biotransformation of racemic **14Z** was carried out with CAL-B (Novozyme 435) and vinyl acetate in TBME obtaining the acylated product (4*E*,15*Z*)-acetate **15Z** and the unreacted alcohol **14Z** being formed in 95 and 65% ee, respectively, after 47% conversion in 2 h at 30°C (Scheme 7.6) [39]. Similarly, racemic **14E** was acylated with CAL-B at 30°C in 2 h, obtaining the remaining alcohol with 81% ee and the acetate **15E** with 94% ee for a 45% conversion.

7.2.1.5 E-4018 (**16**)

Compound **16** is a potent analgesic, the (*R*)-enantiomer being more active than the (*S*)-isomer. The alcohol **17** is an effective building block for the synthesis of **16** and its enzymatic resolution was achieved by a lipase-catalyzed transesterification using PSL, molecular sieves, and vinyl acetate as solvent and acyl donor, in this way the monoacetate (*S*)-**18** was obtained in 55% yield and 96% ee at 60°C (Scheme 7.7) [40].



SCHEME 7.6 Enzymatic resolution of precursors **15Z** and **15E** for the synthesis of a docosa derivative.



SCHEME 7.7 Enzymatic resolution of precursor **17** for the synthesis of **16**.

7.2.1.6 Epothilone A (19)

Pseudomonas AK lipase was used to synthesize the chiral intermediate **20** in the total synthesis of the potent antitumor agent epothilone A [41]. The process was carried out at room temperature using vinyl acetate as acyl donor and hexane as solvent, providing after 72 h the enantiomerically enriched alcohol (*S*)-**20** in 48% isolated yield and 90% ee (Scheme 7.8).

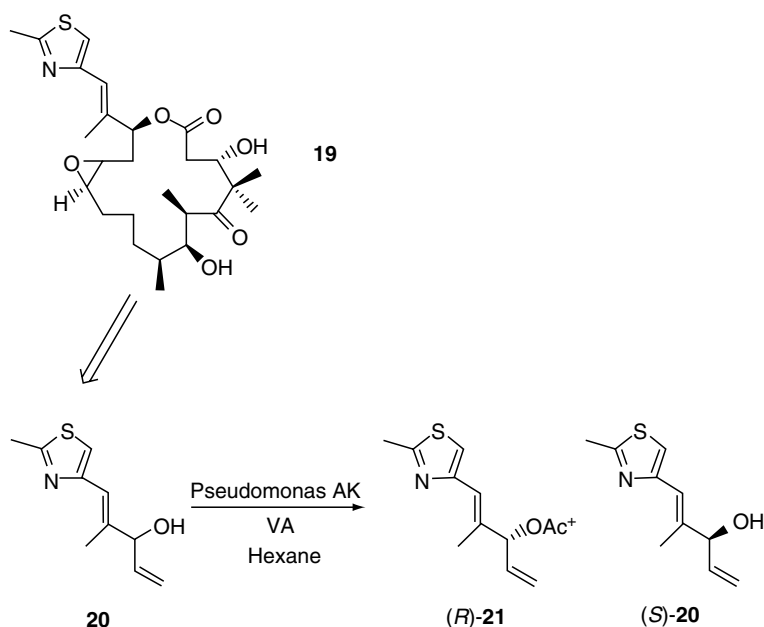
7.2.1.7 Fluoxetine (22), Tomoxetine (23), and Nisoxetine (24)

Compounds **22**, **23**, and **24** are the group of nontricyclic antidepressants that act by inhibiting the uptake of norepinephrine and serotonin. The synthesis of these three compounds was carried out from racemic 3-chloro-1-phenylpropan-1-ol (**25**) by enzymatic transesterification in hexane using vinyl butanoate as acyl donor, and CAL-B as biocatalyst at 30°C (Scheme 7.9) [42]. Alcohol (*S*)-**25** was recovered with 96% ee and 33% isolated yield and acetate (*R*)-**26** with 97% ee and 31% yield.

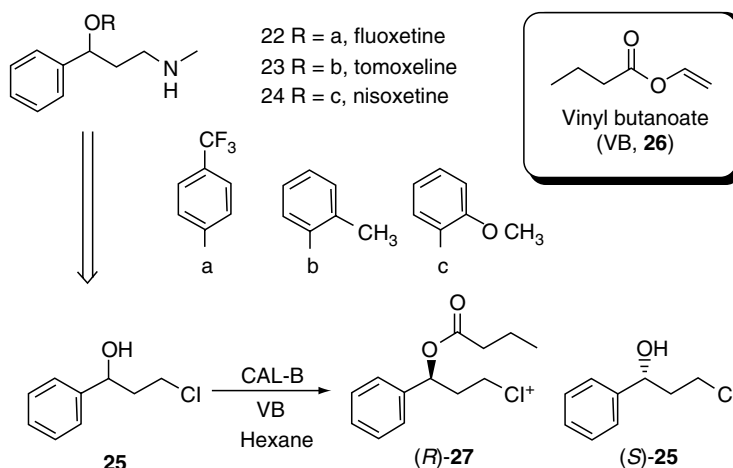
In a different chemoenzymatic approach, these pharmaceutical derivatives were synthesized using 3-hydroxy-3-phenylpropanenitrile **28** as the starting material (Scheme 7.10) [43]. Transesterification of **28** was achieved using both immobilized and nonimmobilized PSL as biocatalyst at 40°C in DIPE. Using immobilized enzyme after 16 h, the (*S*)-alcohol **28** was furnished in 46% yield and 99% ee, same values were obtained for the (*R*)-acetate **29**. Using the free enzyme, the reaction was slower and after 180 h the alcohol was obtained in 46% and the acetate in 44% yields, with ee > 99% for both compounds.

7.2.1.8 Formoterol (30)

The potent β_2 -receptor agonist formoterol (*R,R*)-**30** is on the market as a diastereomeric mixture despite its varying efficacy of stereoisomer. The preparation of the (*R,R*)-stereoisomer



SCHEME 7.8 Enzymatic resolution of precursor **20** for the synthesis of **19**.



SCHEME 7.9 Enzymatic resolution of precursor **25** for the synthesis of **22**, **23**, and **24**.

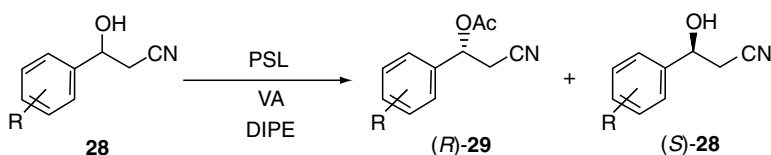
was achieved by enzymatic resolution of (*R*)-bromohydrin **31** (Scheme 7.11) [44]. Immobilized lipase PS-30 from *Pseudomonas cepacia*, vinyl acetate, and TBME were used as solvent, and after 50% conversion at 37°C and 99 h reaction, the acetylated product (*S*)-**32** (48% yield and 86% ee) and the unreacted desired (*R*)-**31** (46% isolated yield and 96% ee) were obtained.

7.2.1.9 HMG CoA Inhibitors

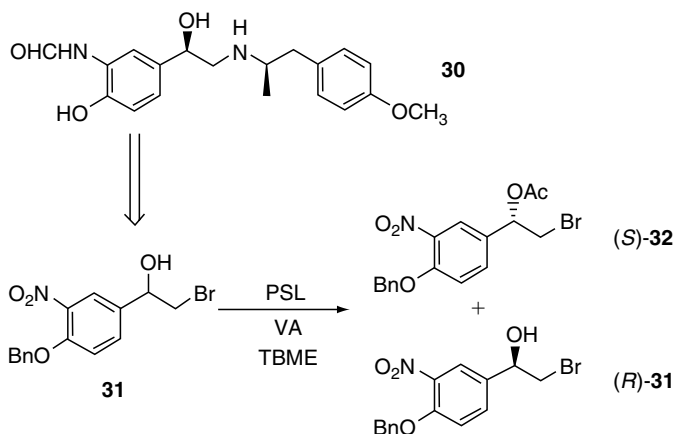
Compound [4-[4 α ,6 β (E)]]-6[4,4-bis(4-fluorophenyl)-3-(1-methyl-1H-tetrazol-5-yl)-1,3-butadienyl]-tetrahydro-4-hydroxy-2H-pyren-2-one, (*R*)-(+)-**33**, is an anticholesterol drug that acts by inhibition of HMG CoA reductase. It was resolved by the lipase-catalyzed stereoselective acetylation of the racemic alcohol using PS-30 lipase in toluene and isopropenyl acetate as acyl donor obtaining the (*R*)-alcohol with ee > 98% and 49% yield (Scheme 7.12) [45]. The industrial scale resolution process was also developed in methyl ethyl ketone at 50 g/L substrate concentration, a reaction yield of 46% and 96% ee was obtained. Furthermore, lipase was immobilized on Accurel PP and the enzyme was reused five times without loss of activity. The enzymatic process was scaled up to a 640 L preparative batch using immobilized PS-30 lipase at 4 g/L of racemic substrate in toluene as a solvent, isolating (*R*)-alcohol in 40% yield with 98.5% ee.

7.2.1.10 β -Lactams

β -Lactam antibiotics have been obtained using an one-step enzymatic process from their corresponding building blocks. Nagai and coworkers developed the enzymatic synthesis



SCHEME 7.10 Enzymatic resolution of precursor **28** for the synthesis of **22**, **23**, and **24**.

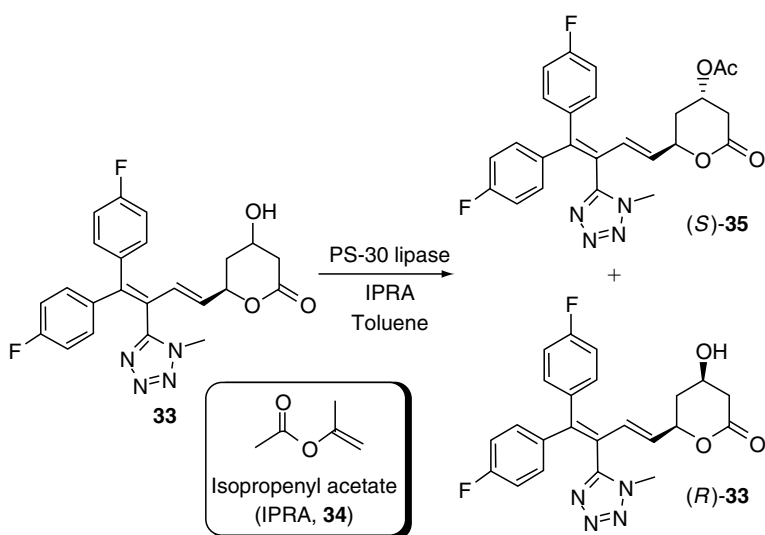


SCHEME 7.11 Enzymatic resolution of precursor **31** for the synthesis of formoterol.

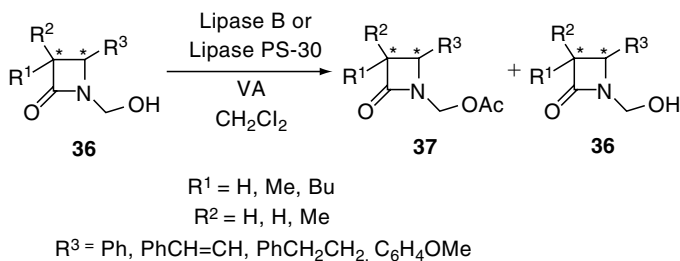
of optically active β -lactams by lipase-catalyzed KR using the transesterification of *N*-hydroxymethyl β -lactam **36** in dichloromethane in the presence of vinyl acetate as acyl donor (Scheme 7.13) [46]. The reaction yields of 35 to 50% and ee of 93 to to 99% were obtained depending on the specific substrate used in this process. Lipase B from *Pseudomonas fragi* and lipase PS-30 were used in these reactions.

7.2.1.11 3-Methylnonacosanol (**38**)

Compound **38** is traditionally used for the treatment of jaundice, enlargement of the liver and spleen, leprosy, and other skin diseases. The (S)-**38** was prepared through the acetate intermediate (S)-**40** obtained by the enzymatic resolution of racemic alcohol **38** (Scheme 7.14) [47,48]. CRL catalyzed the transesterification of **39** using vinyl acetate in DIPE at room temperature. After 5 h and 40% conversion, the acetate (S)-**40** (ee > 96%) and the resolved



SCHEME 7.12 Enzymatic resolution of **33**.



SCHEME 7.13 Enzymatic resolution of **36**.

(*R*)-**39** (71% ee) were produced. A second acetylation of the partially resolved **39** led to the (*R*)-alcohol with 98% ee.

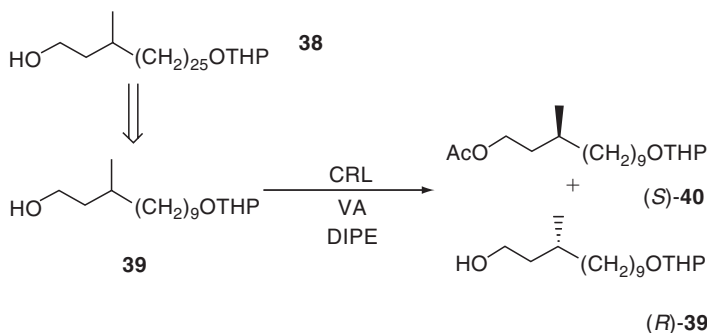
7.2.1.12 Nifenalol (**41**) and Sotalol (**42**)

Drugs bearing a structured unit of 2-amino-1-arylethanol such as **41** and **42** are of great importance as β -adrenergic blockers. They are also used in the therapy of asthma, bronchitis, and congestive heart failure. Among their enantiomers, only (*R*)-(-)-**41** and (*S*)-(+)-**42** are β -adrenergic blockers and are effective in the treatment of cardiovascular diseases. Both **41** and **42** have a common precursor, i.e., 2-bromo-1-(4-nitrophenyl)ethanol (**43**), while their final products have opposite configurations at C-1 (Scheme 7.15).

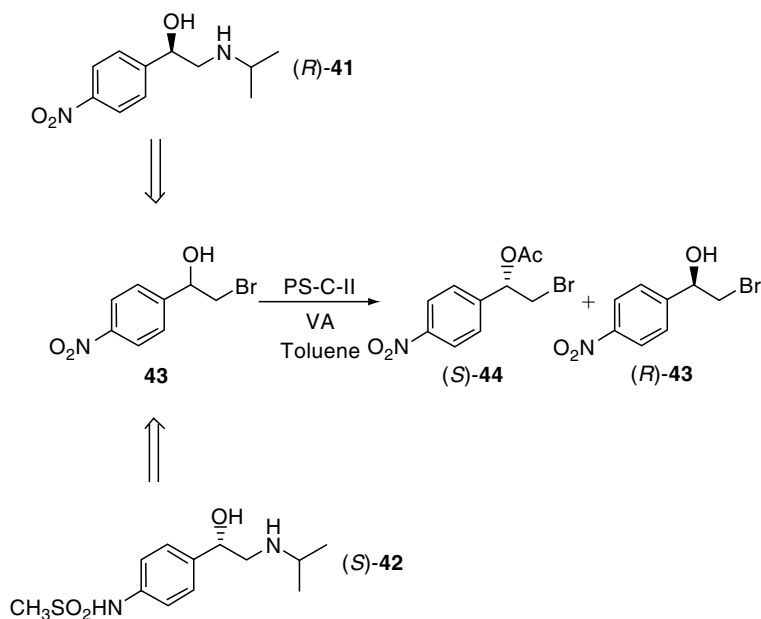
The transesterification reaction of **43** using PS-C-II as biocatalyst, vinyl acetate as acyl donor, and toluene as solvent led to the formation of (*S*)-acetate **44** in 57% yield and (*R*)-alcohol **43** in 42% yield, both compounds obtained with ee > 99% [49].

7.2.1.13 Nilvadipine (**45**)

Nilvadipine is a calcium antagonist of the dihydropyridine group. Owing to its high receptor affinity, nilvadipine blocks *L*-type calcium channels in vascular muscle cells. This leads to prolonged vascular relaxation and lowering of blood pressure. The transesterification of racemic isopropyl 2-hydroxymethyl-1,4-dihydro-6-methyl-4-(3-nitrophenyl)-3-methoxycarbonyl-5-pyridinecarboxylate (**46**) was carried out in acetone using vinyl acetate as acyl donor and PSL as biocatalyst at 40°C obtaining the (*S*)-acetate **47** in 72% ee and 55% yield and the remaining unreacted (*R*)-alcohol in 97% ee and 42% yield after 44 h (Scheme 7.16) [50].



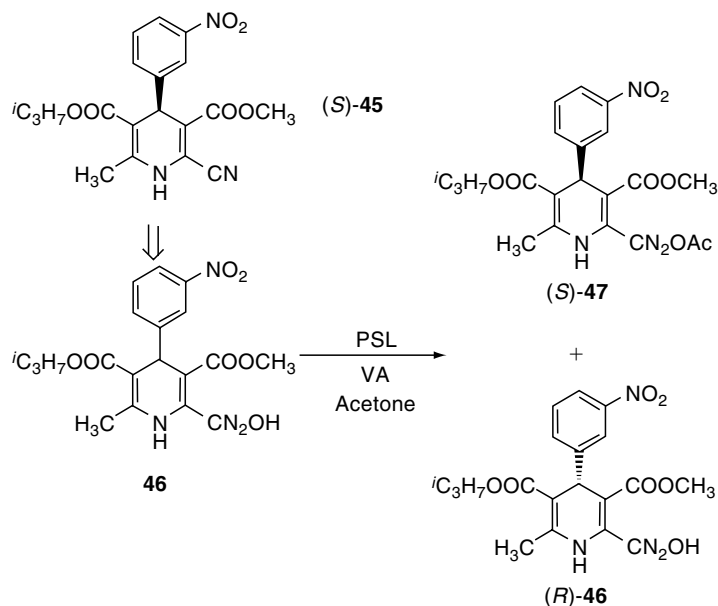
SCHEME 7.14 Enzymatic resolution of **39** for the production of 3-methylnonacosanol.



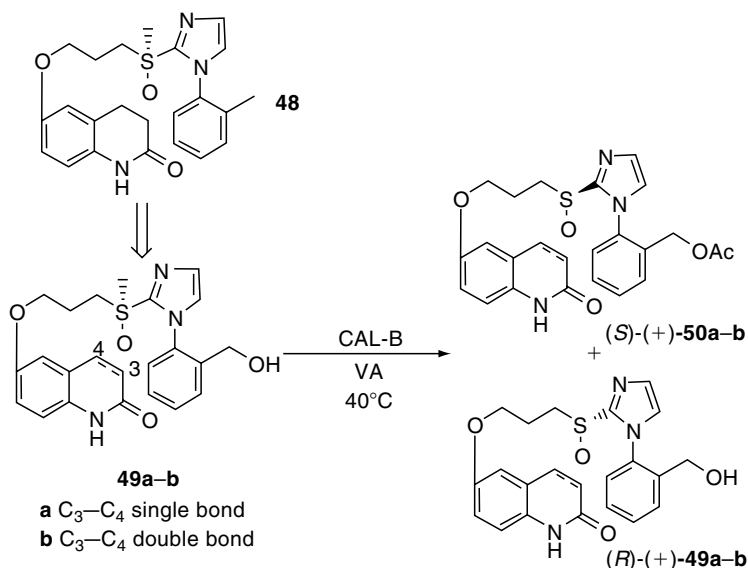
SCHEME 7.15 Enzymatic resolution of **43** for the production of (*R*)-nifenalol and (*S*)-sotalol.

7.2.1.14 OPC-29030 (**48**)

Compound (*S*)-3,4-dihydro-6-[3-(1-*o*-tolyl-2-imidazolyl)sulfinylpropoxy]-2(1*H*)-quinolinone (OPC-29030, **48**) is a platelet adhesion inhibitor that suppresses the production of 12-(*S*)-hydroxyeicosatetraenoic acid in platelets and has been under clinical trials. Metabolites **49a–b**



SCHEME 7.16 Enzymatic resolution of **46** for the production of nilvadipine.



SCHEME 7.17 Enzymatic resolution of **49a-b** for the production of OPC-29030.

were resolved at room temperature using lipase-catalyzed transesterification with CAL-B (Novozyme 435) and vinyl acetate in CH₂Cl₂ (Scheme 7.17) [51]. (*S*)-Acetate **50a** was obtained in 93% ee and (*R*)-alcohol **49a** with 82% after 47% conversion, while (*S*)-acetate **50b** was recovered in 40% isolated yield and 97% ee and (*R*)-alcohol **49b** in 89% ee and 46% isolated yield.

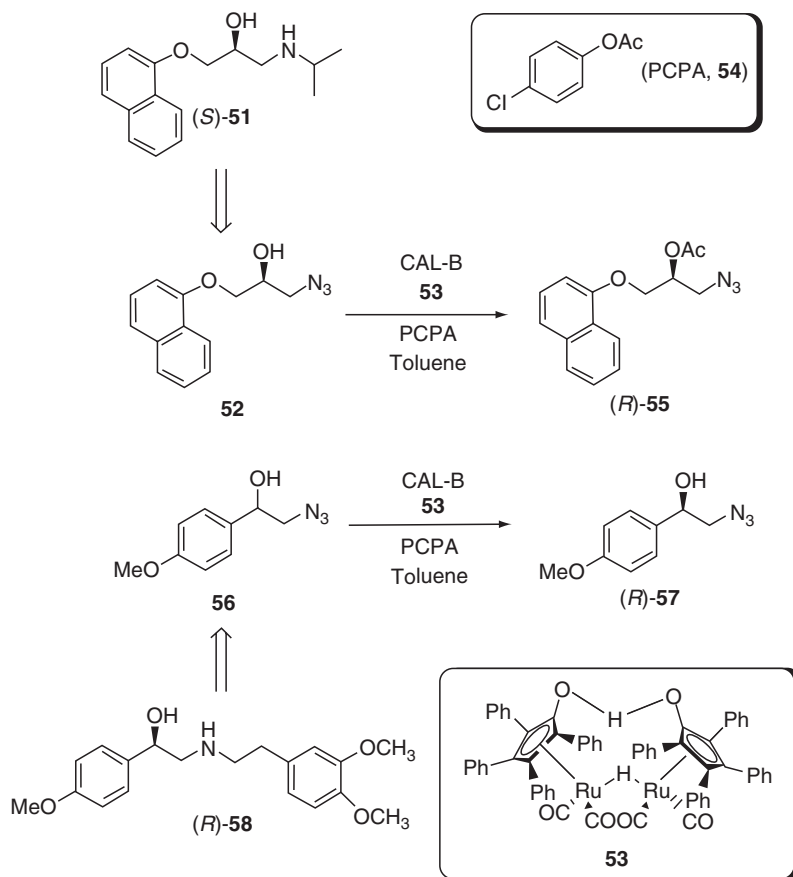
7.2.1.15 Propanolol (**51**)

Propanolol **51** belongs to the group of β -adrenergic blocking agents (β -blockers) of the general structure ArOCH₂CH(OH)CH₂NHR, where Ar is an aryl and R is an alkyl. These compounds are used for the treatment of hypertension and angina pectoris. It has been shown that β -adrenergic receptor blocking activity resides mostly in (*S*)-enantiomers. Synthesis of (*S*)-**51** was achieved by the DKR of (\pm)-**52** using a combination of the ruthenium complex **53** and CAL-B (Novozyme-435) in toluene at 80°C, and *p*-chlorophenyl acetate (**54**) as acyl donor (Scheme 7.18) [52]. The (*R*)-acetate **55** was produced in ee > 99% and 86% isolated yield after 1 d reaction. The enzyme was recycled and used again for another cycle without any loss of activity. Using the same procedure, the racemic **56** was subjected to DKR to produce the (*R*)-acetate **57** (ee > 99%, conversion 92%, and isolated yield 84%) precursor of (*R*)-denopamine (**58**), a potent orally active β_1 receptor agonist for the treatment of heart failure.

The stereoselective preparation of chiral building blocks in the synthesis of **51** can be achieved via acylation of alcohols **59** [53] or **61** [54] in organic solvents using the lipase from *Pseudomonas cepacia* (Scheme 7.19). Using the cyanohydrin **59**, the (*S*)-alcohol was obtained in 96% ee after 56% conversion, while using the chloroalcohol **61** the acetate precursor (*R*)-**62** reached ee > 95% after 47% conversion.

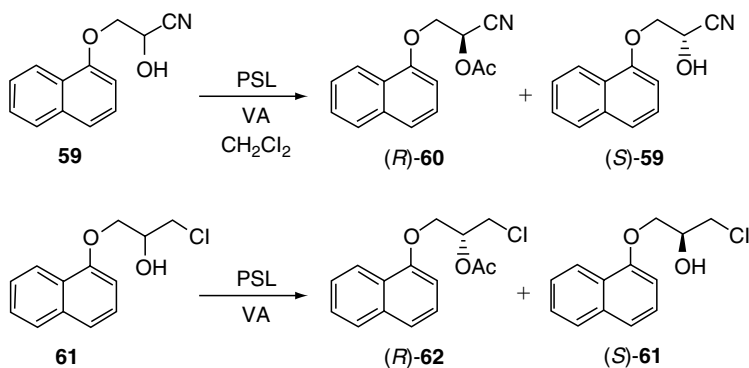
7.2.1.16 Serotonin Antagonism and Uptake Inhibitors

The indanamine derivative MDL 27777A (**63**) is a serotonin uptake inhibitor, and studies showed that (+)-**63** is at least 10 times more active in inhibiting serotonin uptake both *in vitro* and *in vivo* than (–)-**63**. The inhibitor (+)-**63** was synthesized in both “cold” form and

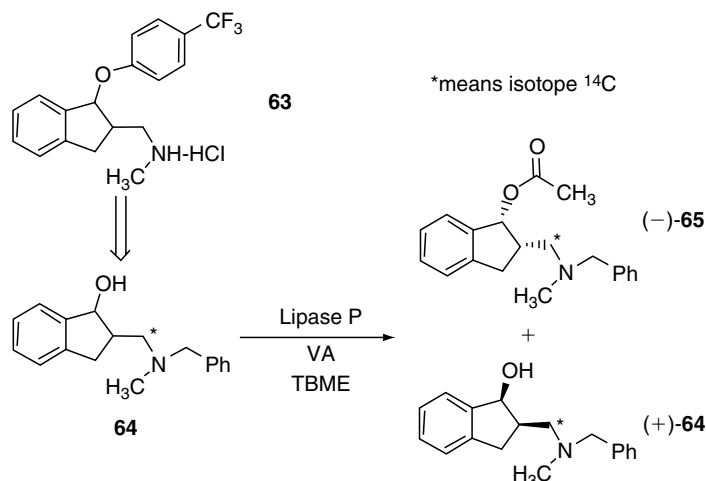


SCHEME 7.18 Dynamic kinetic resolution of **52** and **56**, precursors of (*S*)-propanolol and (*R*)-denopamine.

^{14}C -labeled. Preparation of ^{14}C -**63** is the first example of the use of stereoselective enzymatic acylation in organic solvents for the resolution of radiolabeled compounds [55]. This was achieved using vinyl acetate, TBME, and lipase P as biocatalyst (Scheme 7.20). After 4 d at 22°C, the acetate (–)-**65** (43% yield, 97% ee) and the alcohol (+)-**64** (46% yield, 98% ee) were recovered.



SCHEME 7.19 Enzymatic resolution of **59** and **61**, precursors of (*S*)-propanolol.



SCHEME 7.20 Enzymatic resolution of **64**, precursor of MDL 28618A, [(+)-**63**].

Serotonin antagonist **66**, which was identified as a nonnarcotic analgesic and muscle relaxant, has two chiral centers and therefore exists in the form of four stereoisomers with different biological properties. Resolution of compounds with several chiral centers presents a significant challenge, so **66** was divided into two fragments, the secondary alcohols **67** and **68**, were resolved by enzymatic acylation obtaining four chiral building blocks (*R*)-**69**, (*S*)-**67**, (*R*)-**70**, and (*S*)-**68**, in good yield and excellent optical purity (Scheme 7.21). The simple combination of these blocks gave four different enantiomers of **66** [56].

7.2.2 RESOLUTION OF DIOLS

7.2.2.1 Baclofen (71)

Compound 4-amino-3-(4-chlorophenyl)butanoic acid (**71**) is an analog of γ -aminobutyric acid, which is an inhibitory neurotransmitter that regulates the control of neuronal activity in the central nervous system to regulate several other physiological mechanisms. Unlike GABA, baclofen can cross the blood–brain barrier, and racemic baclofen is widely used as antispasmodic agent. The enantiomers of this compound differ in the pharmacodynamic and toxicological properties; the (–)-enantiomer is not only more active but also more toxic than the (+)-enantiomer.

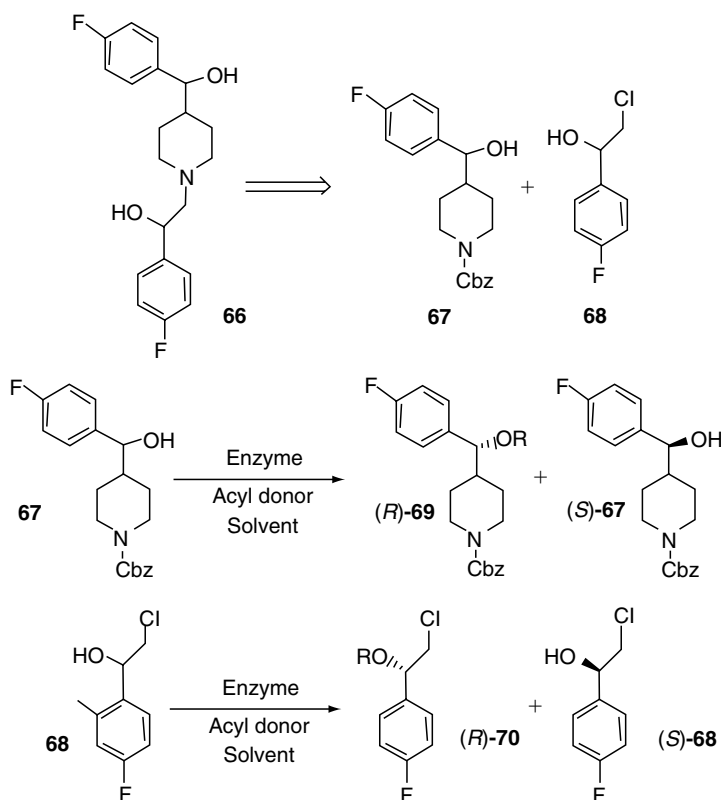
Esterification of 2-(4-chlorophenyl)-1,3-propanediol (**72**) by acetic anhydride in benzene, using porcine pancreas lipase (PPL), gave the hemiester (*S*)-**73** in high ee (>96%) and 93% yield (Scheme 7.22).

7.2.2.2 Camptosar (74)

Camptosar is used in the treatment of ovarian cancer. Sih developed the enzymatic resolution of diol **75** using PSL supported on celite. Using isopropenyl acetate as acyl donor in TBME, (*S*)-**75** was obtained in 40% isolated yield and 99% ee after 1 d reaction at 25°C (Scheme 7.23) [57].

7.2.2.3 Cispentacin (77)

The β -amino acid cispentacin, (1*R*,2*S*)-2-aminocyclopentanecarboxylic acid (**77**), is an antifungal antibiotic. Diol (1*RS*,2*SR*)-**78** was first monosilylated and later the silyloxy alcohol

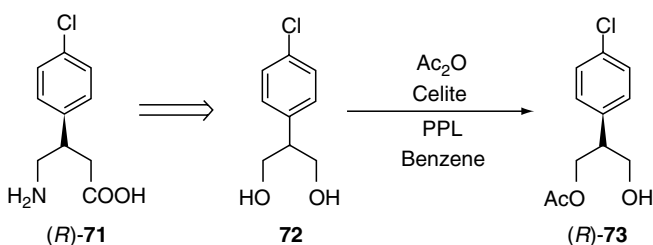


SCHEME 7.21 Enzymatic resolution of **67** and **68**, precursors of **66**.

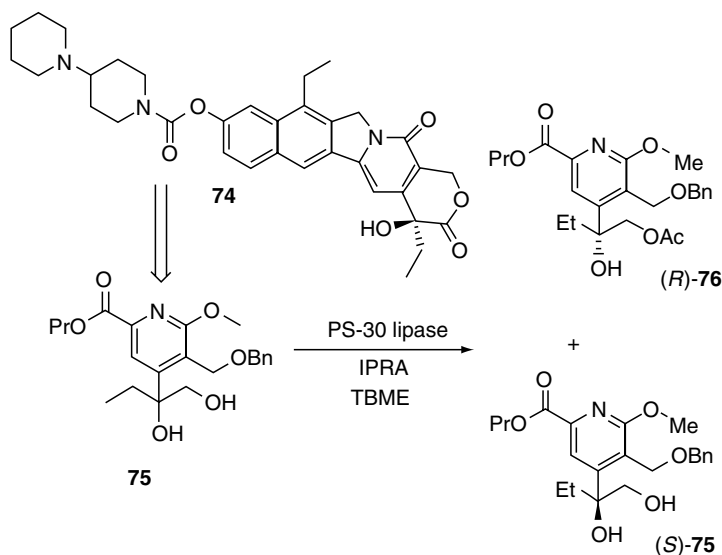
(1*RS*,2*SR*)-**79** selectively acetylated with vinyl acetate using PSL in TBME affording after 40% conversion the acetate (1*R*,2*S*)-**80** with an ee of 94% (Scheme 7.24). The remaining enantiomerically enriched silyloxy alcohol (1*S*,2*R*)-**79** with an ee of 51% was subjected to a second transesterification that was terminated at 20% conversion (60% from start) and yielded a further fraction of (1*R*,2*S*)-**80** with an ee of 98% and the remaining alcohol (1*S*,2*R*)-**79** with 99% ee [58].

7.2.2.4 ϵ -Hydroxytramadol (**81**) and δ -Hydroxytramadol (**82**)

Tramadol (**83**) and its modified analogs ϵ -hydroxytramadol (**81**) and δ -hydroxytramadol (**82**) exhibit interesting pharmacological properties as analgesics. The *Candida rugosa*

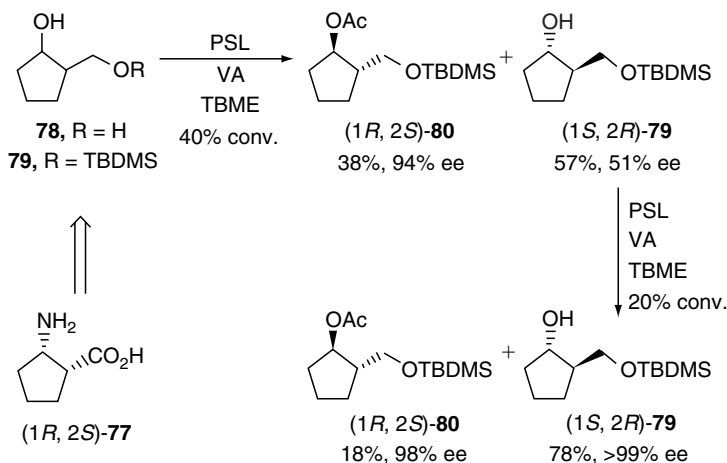


SCHEME 7.22 Enzymatic resolution of **72**, precursors of baclofen.

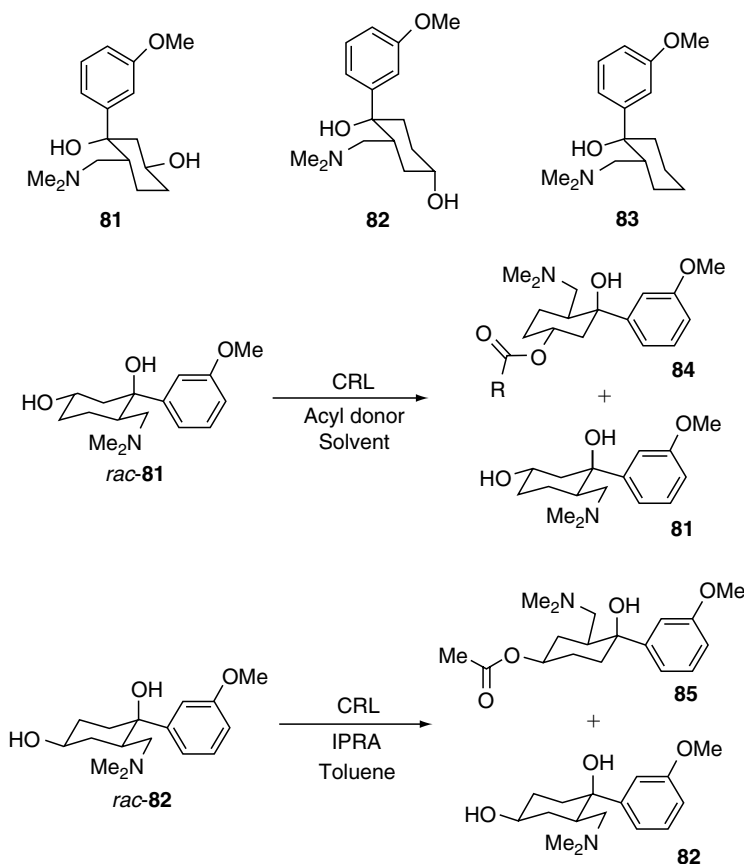


SCHEME 7.23 Enzymatic resolution of **75** for the synthesis of camptosar.

lipase (CRL)-catalyzed transesterification of racemic **81** was studied using vinyl acetate as acyl donor and solvent or isopropenyl acetate as acyl donor and toluene as solvent (Scheme 7.25) [59]. In the first case, after 7 d at room temperature and 56% conversion, alcohol **81** was obtained with 97% ee and ester **84** with 91% ee. When isopropenyl acetate was used as acyl donor, alcohol was obtained in 60% ee and ester **77** in 99% ee after 5 d of reaction at 34% conversion. Having obtained favorable results with the transesterification of **81**, the CRL-catalyzed acylation of *rac*-**82** was investigated. However, the acylation of **82** was not as selective as that of *rac*-**81**. Using isopropenyl acetate (IPRA) and toluene at room temperature the conversion reached 25% after 16 d affording the ester **85** in 68% ee and the alcohol **82** in 87% ee.



SCHEME 7.24 Enzymatic resolution of **79** for the synthesis of cispentacin.



SCHEME 7.25 Enzymatic resolution of ϵ -hydroxytramadol and δ -hydroxytramadol.

7.2.2.5 Leustrodiscin B (86)

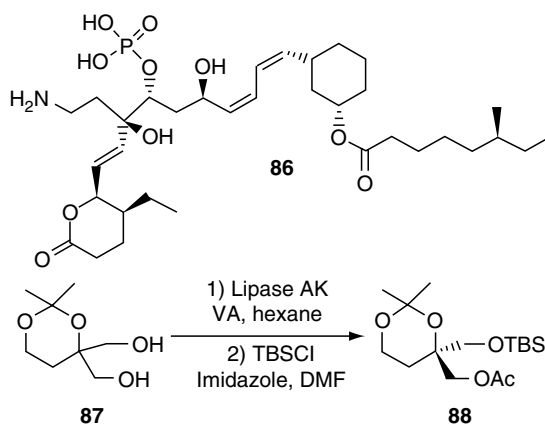
Leustrodiscin B is a potent colony-stimulating factor inducer, and it induces cytokine productions via NF- κ B activation at the transcription level as well as at the posttranscriptional level. Desymmetrization of *meso*-diol **87** at room temperature with lipase AK in hexane using vinyl acetate as acyl donor gave the corresponding (*R*)-acetate that was immediately protected to form **88** in an overall yield of 86 and 90% ee (Scheme 7.26) [60].

7.2.2.6 Propanolol (51)

Therapeutic properties of (*S*)-propanolol have been described in Section 7.2.1 “Resolution of Monoalcohols.” Lipase P from *Pseudomonas fluorescens* catalyzed the stereoselective acylation of the *meso*-diol **89** using vinyl acetate. The (*S*)-**90** was recovered in 92% isolated yield with 94% ee, after 4 h reaction at 8°C (Scheme 7.27) [61].

7.2.2.7 SCH 51048 (91)

The tetrahydrofuran **91** is an antifungal agent with therapeutic potential against a variety of systemic fungal infections in normal and immunocompromised infection models. The synthesis of **91** was achieved using an enzymatic desymmetrization of the homoallylic diol **92**



SCHEME 7.26 Enzymatic resolution of **87** for the synthesis of leustroduscin B.

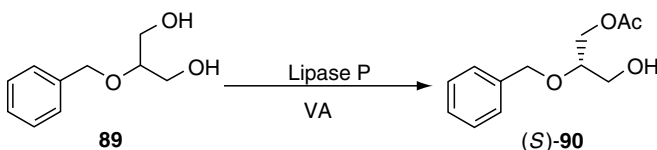
with CAL-B (Novozyme 435) to provide the desired (*S*)-monoacetate **93** in 71% yield and 98% ee using vinyl acetate and MeCN at 0°C (Scheme 7.28) [62]. These conditions allowed to prepare up to 30 kg batches of (*S*)-**93**, and the catalyst reusability was demonstrated over six cycles without major loss of activity.

7.2.2.8 Sobrerol (**94**)

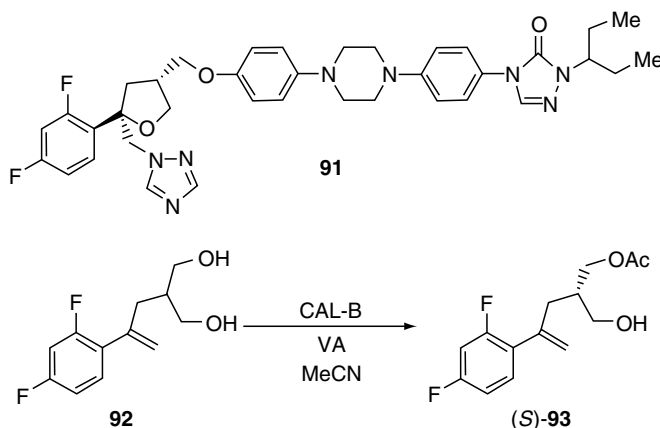
Compound (\pm)-*trans*-5-(1-hydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-ol [(\pm)-*trans*-sobrerol, **94**] is a mucolytic drug, and in spite of its differences in the pharmacological activity between the (+) and (–) forms, it is produced and marketed as racemate. Immobilized PSL selectively acetylated **94** using *tert*-amyl alcohol as solvent and vinyl acetate as acylating agent, the monoacetate (–)-**95** and the diol (+)-**94** were obtained in 99% ee after 50% conversion in 2 h reaction at 45°C (Scheme 7.29) [63].

7.2.2.9 Spyrotryprostatins A (**96**) and B (**97**), (–)-Physostigmine (**98**), and (–)-Esermethole (**99**)

Oxindole and indoline skeletons having a stereogenic quaternary carbon center at the C-3 position are found in many biologically important indole alkaloids, such as spyrotryprostatins A and B, (–)-physostigmine, and (–)-esermethole. Kita and coworkers reported the desymmetrization of diols **100a–d** using lipase OF and 1-ethoxyvinyl-2-furoate as acyl donor in mixtures of *i*Pr₂O:THF at 30°C obtaining the corresponding monoacetates **102a–d** in good yields and high ee (Table 7.1 and Scheme 7.30) [64,65].



SCHEME 7.27 Enzymatic resolution of **89** for the synthesis of (*S*)-propanolol.



SCHEME 7.28 Enzymatic desymmetrization of **92** for the synthesis of retiferol.

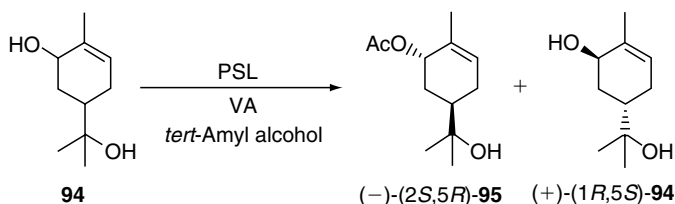
7.2.3 RESOLUTION OF POLYALCOHOLS

7.2.3.1 Glycerol Derivatives

Tumor inhibitory activity of glycosylglycerols and glycolipids is demonstrated on the basis of their *in vitro* and *in vivo* antitumor promoting effect on Epstein–Barr virus, early antigen activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate. PSL catalyzed the regioselective transesterification of the primary hydroxyl group of **104** in THF using different trifluoroethyl esters obtaining the 1,3-di-*O*-benzyl-2-*O*-(6-*O*-acyl- β -D-glucopyranosyl)-glycerols **105** in good yields (Scheme 7.31) [66]. To obtain the 3-esters **107**, CAL-B was used in THF using the appropriate trifluoroethyl ester affording 3-*O*-acyl-2-*O*-(2,3,4,6-tetra-*O*-chloroacetyl- β -D-glucopyranosyl)-glycerols in good diastereoselectivity and yields.

7.2.3.2 Inositol Phosphates

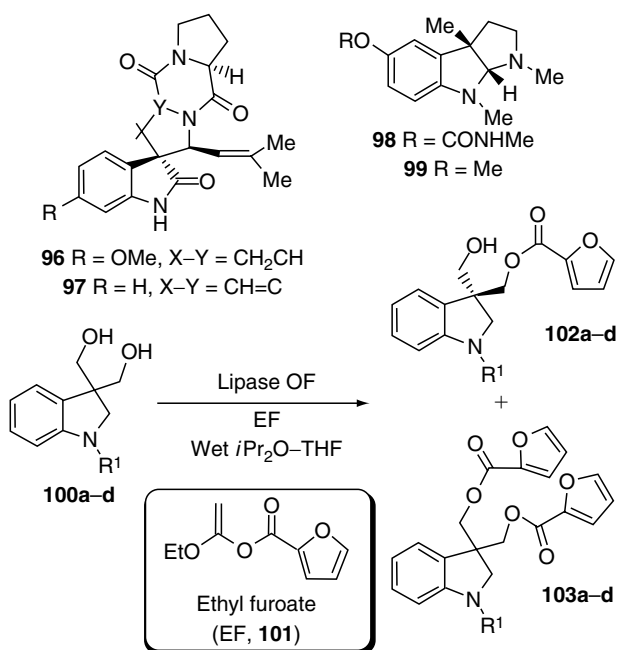
A significant number of physiological processes in differentiated higher cells are closely linked with inositol metabolism. Important examples are the activation of thrombocytes in the blood clotting process, hormonal signal transduction, signal transformation, contraction of muscles, control of cell proliferation, etc. D-*myo*-inositol phosphate **108** was synthesized by a short route from D-1-acetoxy-4,6-di-*O*-benzyl-*myo*-inositol (**110**), which was easily obtained by an initial highly regio- and enantioselective acylation of 4,6-di-*O*-benzyl-*myo*-inositol (**109**) catalyzed by PSL (Scheme 7.32) [67]. CAL-B showed good selectivity toward racemic 2,6-di-*O*-benzyl-*myo*-inositol (**111**) using vinyl acetate as acyl donor yielding 49% of the unconverted inositol derivative (–)-2,6-di-*O*-benzyl-*myo*-inositol with 99% ee and monoacetylated product **112** with 49% yield, which was easily converted to (+)-2,6-di-*O*-benzyl-*myo*-inositol by



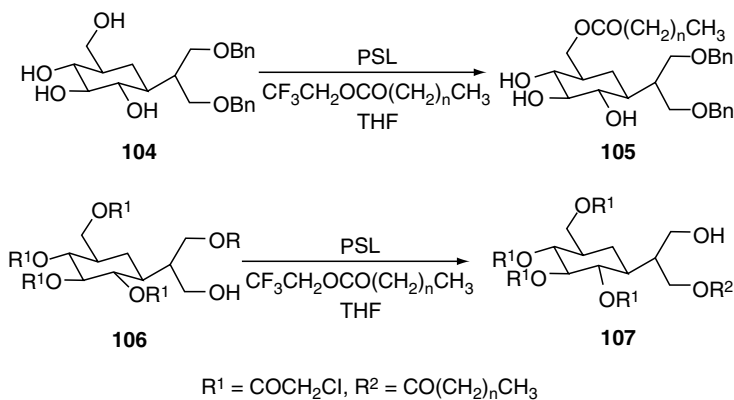
SCHEME 7.29 Enzymatic resolution of (±)-*trans*-sobrerol (**94**).

TABLE 7.1
Enzymatic Desymmetrization of Diols 100a–d

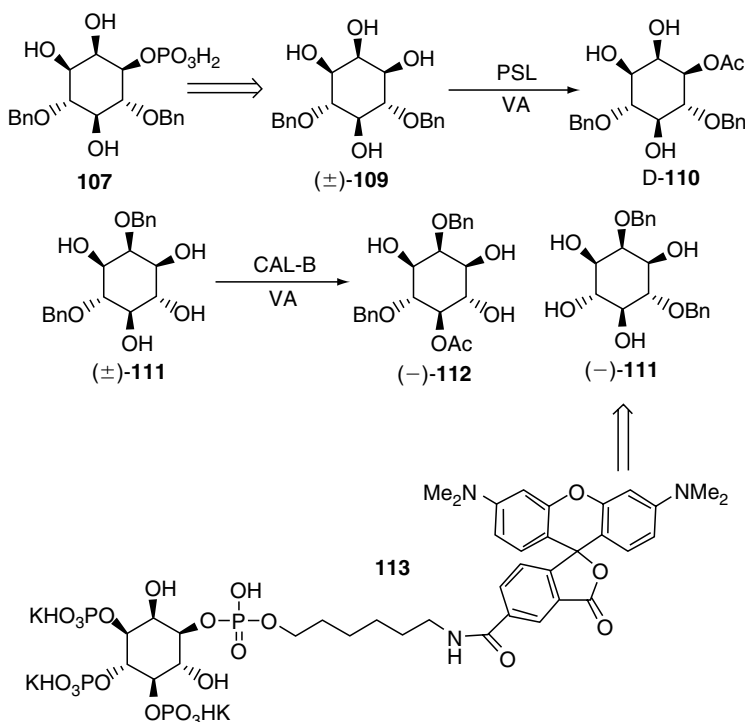
Entry	Compound	R ¹	R ²	<i>i</i> Pr ₂ O:THF	<i>t</i> (h)	102 (ee [%])	102 (Yield [%])
1	100a	Me	H	5:1	20	79	60
2	100b	Boc	H	100:0	20	97	68
3	100c	Boc	5-OMe	5:1	3	98	77
4	100d	Boc	6-OMe	5:1	19	91	79



SCHEME 7.30 Enzymatic desymmetrization of diols **100a–d** for the synthesis of **96–99**.



SCHEME 7.31 Selective modifications of glycerol derivatives.



SCHEME 7.32 Resolution of **109** and **111**, precursors of inositol derivatives.

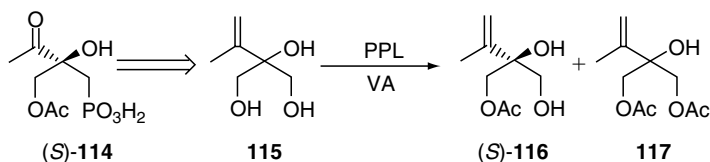
chemical hydrolysis [68]. Pure (–)-2,6-di-*O*-benzyl-*myo*-inositol was also used for the synthesis of 5'-TAMRA-labeled (P1-tethered)-D-*myo*-inositol-3,4,5-triphosphate (**113**), a biochemical agent for binding assays to identify and characterize pharmaceutically relevant proteins.

7.2.3.3 Phosphonotrixin (**114**)

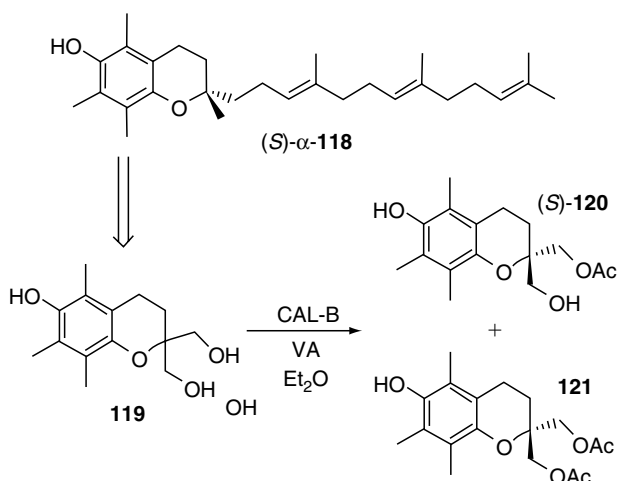
Phosphonotrixin, a secondary metabolite, has attracted interest due to its antibiotic and herbicidal properties. The synthesis of (*S*)-(–)-**114** was achieved through desymmetrization of triol **115** occurred in vinyl acetate and catalyzed by PPL [69]. After 5 h at room temperature, the monoacetate **116** was obtained in 88% isolated yield and the diacetate **117** in 7% yield (Scheme 7.33).

7.2.3.4 (*S*)- α -Tocotrienol (**118**)

Tocotrienols inhibit cholesterol biosynthesis by posttranscriptional suppression of β -hydroxy- β -methyl-glutaryl-coenzyme A reductase activity. Also, tocotrienols are effective antioxidants and inhibit the oxidation of low density lipoproteins associated with coronary heart disease.



SCHEME 7.33 Desymmetrization of **115**, precursor of (*S*)-phosphonotrixin.



SCHEME 7.34 Enzymatic desymmetrization of **119**, precursor of (*S*)- α -tocotrienol.

In addition, they have shown anticarcenogenic and neuroprotective properties. The desymmetrization of triol **119** with vinyl acetate in the presence of CAL in Et₂O gave enantiomerically pure (ee > 98%) monoester **120** in 60% yield and the corresponding achiral diester **121** in 27% isolated yield after 2 h reaction (Scheme 7.34) [70].

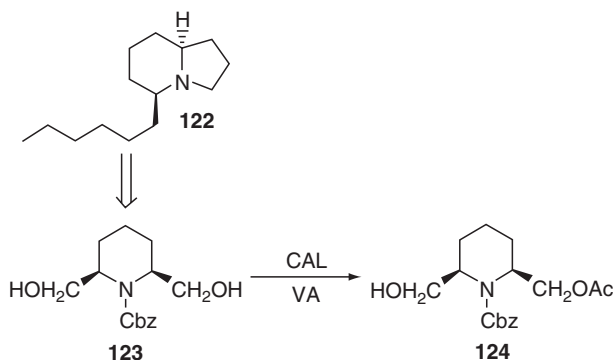
7.3 ENZYMATIC ACYLATION OF AMINOALCOHOLS

7.3.1 (5*S*,9*S*)-(+)-INDOLIZIDINE 209D (**122**)

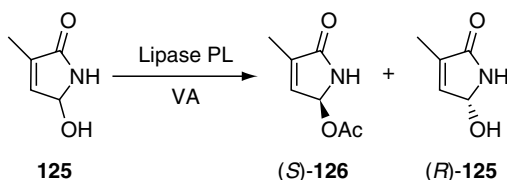
All four possible stereoisomers of indolizidine 209D are potent blockers for nicotinic acetylcholine receptors. Chênevert and coworkers reported the enzyme-catalyzed transesterification of diol **123** using CAL in vinyl acetate at room temperature to give optically active monoester **124** in 95% ee and 80% yield after 3 h reaction (Scheme 7.35) [71].

7.3.2 JATROPHAM (**125**)

(*R*)-(-)-5-hydroxy-3-methyl-3-pyrrolidin-2-one, (*R*)-**125** is an antitumor alkaloid that has been synthesized through the KR of the racemate using lipase PL as biocatalyst and vinyl acetate as acyl donor and solvent at 25°C, isolating the (*R*)-alcohol in 35% yield with 98% ee along with the acetate (*S*)-**126** in 53% yield with 50% ee (Scheme 7.36) [72].



SCHEME 7.35 Enzymatic desymmetrization of **123**, precursor of (5*S*,9*S*)-(+)-indolizidine 209D.



SCHEME 7.36 Kinetic resolution of jatropham.

7.3.3 NORPHENYLEPHRINE (**127**) AND OCTOPAMINE (**128**)

(*R*)-(–)-Noradrenaline **129** is a natural catecholamine that acts as an α -adrenoceptor agonist and also shows activity toward β -receptors. Norphenylephrine (**127**) and octopamine (**128**) are structural analogs of **129** that have similar properties. The synthesis of enantiomerically pure **127** and **128** were possible through the resolution of derivatives **130a–b** using butanoic acid in the presence of PSL in a mixture of toluene/THF as solvent (Scheme 7.37) [73]. *N*-Boc and *N*-Fmoc derivatives of octopamine were also resolved using butanoic anhydride instead of butanoic acid.

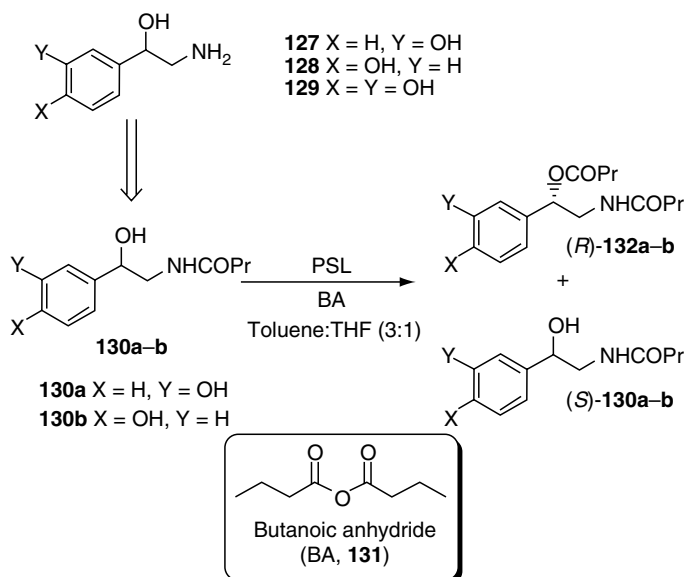
7.4 ENZYMATIC ACYLATION OF AMINES

In this section, we have included separately the resolution of amines, mainly primary amines and 1,2-diamines.

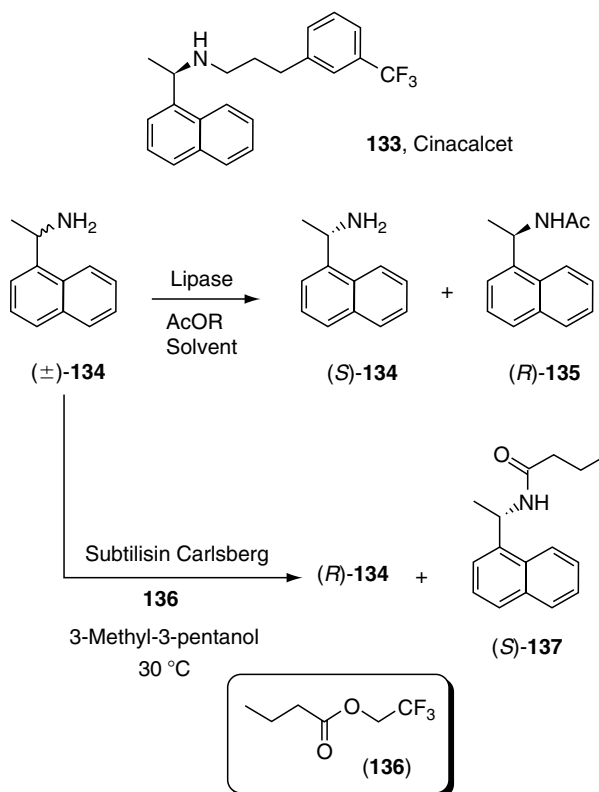
7.4.1 RESOLUTION OF AMINES

7.4.1.1 Cinacalcet (**133**)

Cinacalcet-HCl is an oral calcimimetic drug, which has received the FDA approval in 2004. It is used in the treatment of hyperparathyroidism and for the preservation of bone density in patients with kidney failure or hypercalcemia due to cancer [74]. A precursor of **133** is the



SCHEME 7.37 Enzymatic resolution of **130a–b**, precursors of norphenylephrine and octopamine.



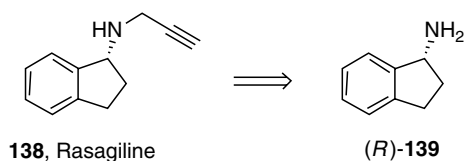
SCHEME 7.38 Resolution of (±)-1-(1-naphthyl)ethylamine (**134**).

optically active (*R*)-1-(1-naphthyl)ethylamine [(*R*)-**134**] [75] or its amide derivative (*R*)-**135**, which has been obtained by enzymatic resolution of (±)-**134** (Scheme 7.38). When CAL-B was used as catalyst, results were greatly dependent on the acyl donor and the solvent used. Low and moderate enantiomeric ratios were obtained using ethyl acetate as the acyl donor (*E* = 5 and 24 with ethyl acetate and diethyl ether as solvents, respectively) [76,77]. However, excellent results were obtained with isopropyl acetate [78] in 1,2-dimethoxyethane and (±)-1-phenylethyl acetate [79] in 1,4-dioxane (*E* > 200 in both cases), allowing the isolation of the acetamide (*R*)-**135** with ee > 99%. In addition, when the acetylation of (±)-**134** was carried out with *Pseudomonas aeruginosa* lipase, ethyl acetate as acyl donor, and TBME as solvent, (*R*)-**135** was obtained with ee > 99% [80].

The protease subtilisin Carlsberg has also been used to catalyze the resolution of (±)-**134** with different activated esters such as 2,2,2-trifluoroethyl butyrate (**136**) [81] and methacrylate [82], or cianomethyl pent-4-enoate [83] and 3-methyl-3-pentanol as solvent. In all the cases, the enzyme exhibited a high enantioselectivity (*E* > 100) toward the (*S*) enantiomer, the enantiopreference being opposite to that shown by the lipases. The resolution of (±)-**134** with 2,2,2-trifluoroethyl butyrate (Scheme 7.38) was scaled up to 1.6 kg of substrate using a continuous-flow column bioreactor containing subtilisin immobilized on glass beads [84]. Thus, 560 g of pure (*R*)-1-(1-naphthyl)ethylamine with 99% ee was prepared.

7.4.1.2 (*R*)-*N*-Propargyl-1-Aminoindan: Rasagiline (**138**)

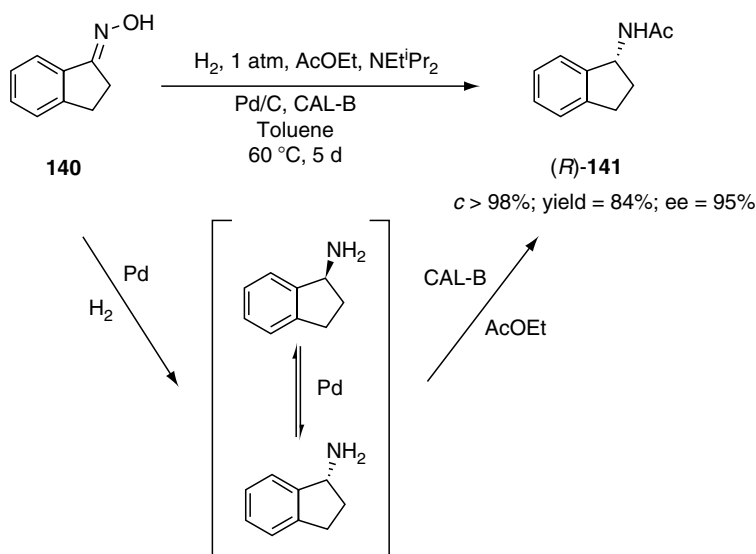
Amine **138** and some of its derivatives have shown to be highly selective and potent inhibitors of the B form of monoamine oxidase, in contrast the levorotatory enantiomer is inactive.



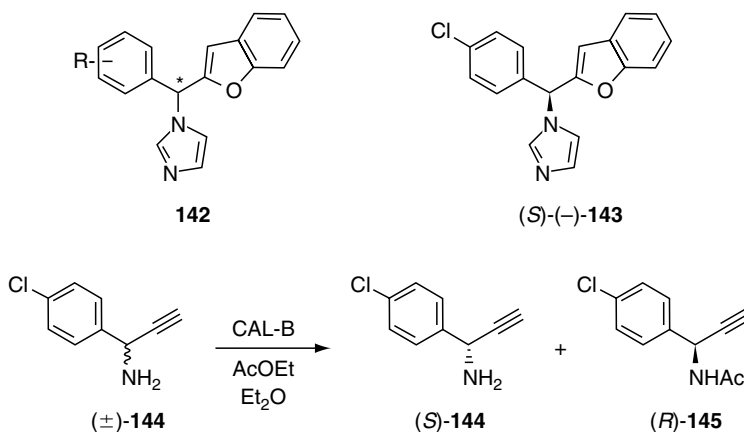
SCHEME 7.39 Retrosynthesis of rasagiline **138**.

These compounds have shown to be useful in the treatment of Parkinson's disease, memory disorders, dementia of the Alzheimer type, depression, and the hyperactive syndrome in children. (*R*)-*N*-Propargyl-1-aminoindan (**138**) has been easily prepared from (*R*)-1-aminoindan [(*R*)-**139**] and propargyl chloride (Scheme 7.39) [85].

Different hydrolytic enzymes have been tested in the resolution of (\pm)-**139**. Amidation of (*R*)-**139** was catalyzed with aminoacylase I from *Aspergillus melleus* [86] and penicillin acylase from *Alcaligenes faecalis* [87] using methyl methoxyacetate and phenylacetamide as acyl donors, respectively, but with very low enantioselectivities ($E \leq 9$). In contrast, subtilisin was very efficient and, as described above for (*R*)-**134**, the aminolysis of 2,2,2-trifluoroethyl butyrate and (\pm)-**139**, scaled up to 330 g of racemic amine, yielded the unreacted (*R*)-1-aminoindan with high yield (132 g, 40%) and ee > 98% [84]. CAL-B also was an excellent catalyst for the resolution of (\pm)-**139** when allyl pent-4-enoate and dibenzyl carbonate were used as acyl and alkoxycarbonyl donors, respectively. In both cases, the lipase was selective toward the (*R*) enantiomer of the amine catalyzing the formation of the corresponding (*R*)-pent-4-enoamide (ee > 99%) [88] and (*R*)-benzyl carbamate (ee = 95%) [89]. This lipase has also been applied in an interesting process combining enzyme and metal catalysis (Scheme 7.40). The starting material was the readily available ketoxime **140** and the catalysts were Pd and CAL-B. Thus, the coupling of the Pd-catalyzed reduction of the ketoxime **140** and the subsequent Pd and CAL-B catalyzed DKR of the resulting racemic amine, afforded acetamide (*R*)-**141** with very high chemical and optical yields [90]. In this process, an additive such as *N*-ethyl-diisopropylamine was required to suppress the reductive deamination of the amine.



SCHEME 7.40 Asymmetric transformation of **140** into (*R*)-**141**.



SCHEME 7.41 Resolution of (±)-**144**.

7.4.1.3 1-[(2-Benzofuranyl)phenylmethyl]imidazoles **142**

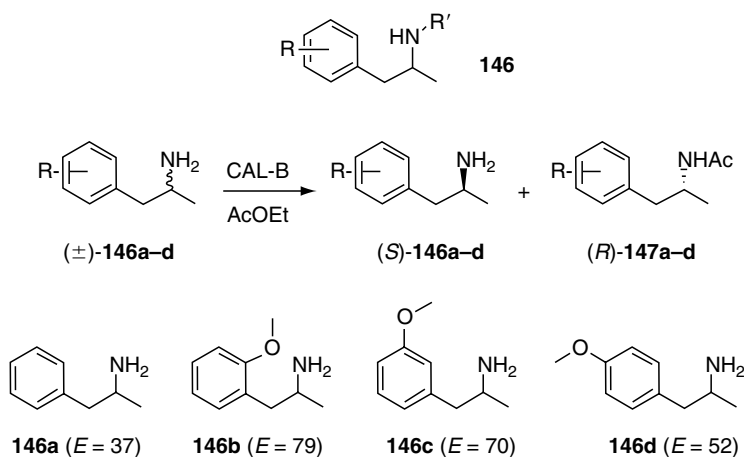
Imidazoles **142** are a new class of potent aromatase inhibitors that show promise as chemotherapeutic agents for the treatment of estrogen dependent tumors. In particular, the 4-chloro analog **143** has shown activity either *in vitro* or *in vivo*, the (+) form being 15-fold more active than its counterpart. A chemoenzymatic synthesis of both enantiomers of **143** has been developed starting from racemic 1-(4-chlorophenyl)-2-propynylamine [(±)-**144**] [91]. Resolution of (±)-**144** was carried out by CAL-B catalyzed acetylation using ethyl acetate as the acyl donor and diethyl ether as the solvent (Scheme 7.41). After 25 h reaction at room temperature, the remaining amine (*S*)-**144** and the converted acetamide (*R*)-**145** were isolated with ee of 98% and >98%, respectively (*E* > 200). Conventional hydrolysis of (*R*)-**145** yielded the amine (*R*)-**144** that was transformed into (*S*)-(-)-**143** (ee = 97.5%) by a three-step synthesis. Analogously, (*S*)-**144** was converted into (*R*)-(+)-**143** in 40 to 45% overall yield in three steps.

The enzymatic resolution has also been efficiently applied to other (±)-1-aryl-2-propynylamines (R = H, 4-F, 3-F, and 3-Me) [92], precursors of imidazoles **142**.

7.4.1.4 1-Arylpropan-2-Amines (**146**): Amphetamine and Derivatives

Amines **146** possess central and peripheral stimulant activity and suppress appetite. The presence of methyl groups on the nitrogen atom and methoxy groups on the aromatic ring is known to increase the effects of the drug. In addition, the stereochemistry of these amines has a great influence on their pharmacological properties. Thus, (*S*)-(+)-1-phenylpropan-2-amine [(*S*)-(+)-amphetamine, dexedrine] has greater pharmacological activity as stimulant [93] and hyperthermic [94] agent than its (*R*)-(-)-enantiomer.

Both enantiomers of the amphetamine (**146a**) and the isomeric *o*-, *m*-, and *p*-methoxyamphetamines (**146b–d**) have been prepared with very high ee by CAL-B catalyzed resolution of the corresponding racemic amines using ethyl acetate as acyl donor and solvent [95]. The unreacted amines (*S*)-**146a–d** and the converted acetamides (*R*)-**147a–d** were easily separated by selective extraction. Enantioselectivity of these reactions was from moderate to high (Scheme 7.42), allowing (*S*)-amines to be obtained with very high ee at 50% conversion. (*R*)-Acetamides, isolated from the enzymatic reactions with ee 82% to 89%, were finally obtained with ee > 98% after recrystallization from hexane–chloroform. The use of a catalytic amount of triethylamine in the reaction of (±)-**146d** slightly improved the enantioselectivity



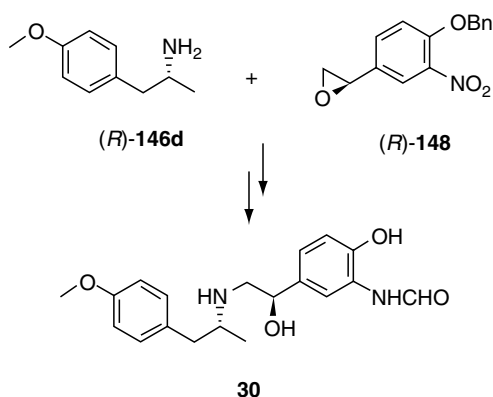
SCHEME 7.42 Resolution of amphetamine and the isomeric methoxyamphetamines.

($E=66$) and the reaction rate [44]. The enantiomerically pure acetamide (R)-**147d** was hydrolyzed and the resulting (R)-**146d** was used in the synthesis of the most active stereoisomer of formoterol (**30**) (Scheme 7.43). The epoxide (R)-**148** was obtained from bromohydrin (R)-**31**, prepared by enzymatic resolution (Scheme 7.11).

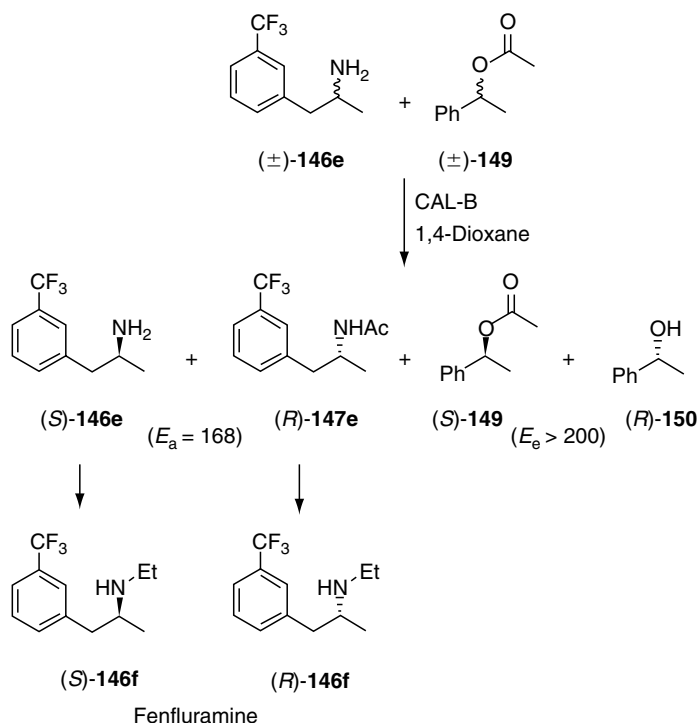
Fenfluramine **146f** (Scheme 7.44) is also an amphetamine derivative with anorectic properties but without stimulant effects. Pharmacological studies have revealed that (S)-**146f** is the active isomer and (R)-**146f** is responsible for the adverse effects [96]. In addition, norfenfluramine **146e** is the main metabolite of **146f** and also its synthetic precursor [97]. Resolution of (\pm)-**146e** has been efficiently accomplished by enantioselective CAL-B catalyzed aminolysis reaction using (\pm)-1-phenylethyl acetate [(\pm)-**149**] as acylating agent and 1,4-dioxane as solvent (Scheme 7.44). Very high enantioselectivities were obtained for amine (E_a) as well as ester (E_e) [79].

7.4.1.5 Mexiletine (151)

1-(2,6-Dimethylphenoxy)propan-2-amine (**151**, mexiletine) is an antiarrhythmic agent, the (R)-enantiomer is more potent for experimental arrhythmias and in binding studies on



SCHEME 7.43 Synthesis of (R,R)-formoterol (**30**).

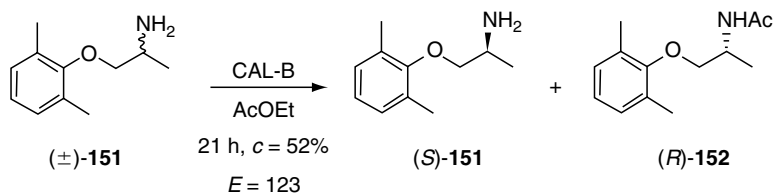


SCHEME 7.44 Chemoenzymatic preparation of fenfluramine (**146f**).

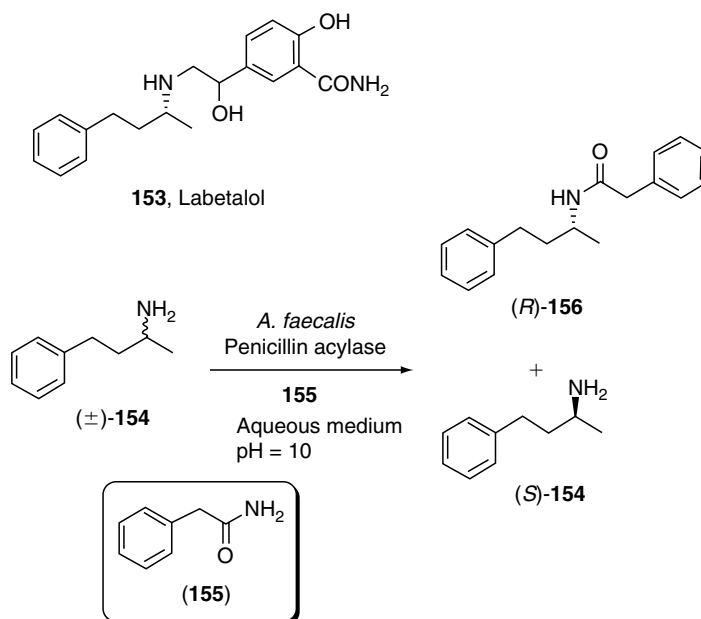
cardiac sodium channels than its (*S*)-counterpart [98]. Using the enzymatic method described for amphetamines (Scheme 7.42), (*S*)-**151** (ee = 99%) and (*R*)-acetamide **152** (ee = 92%) were prepared (Scheme 7.45). A simple recrystallization of the (*R*)-acetamide allowed its isolation in enantiopure form [95].

7.4.1.6 Labetalol (**153**)

Labetalol (Scheme 7.46) is an antihypertensive agent [99], the most active stereoisomer being the divelalol with *R,R* configuration. A building block of this compound is (*R*)-4-phenylbutan-2-amine [(*R*)-**154**], which has been prepared by CAL-B catalyzed amidation of (\pm)-**154** with ethyl acetate. The lipase catalyzed the formation of the corresponding (*R*)-acetamide (*c* = 51%, ee = 86%) with moderate enantioselectivity (*E* = 41), but its recrystallization from hexane–chloroform increased the ee up to 99% [95]. The resolution of (\pm)-**154** was also carried out by acylation reaction catalyzed by penicillin acylase from *Alcaligenes faecalis* in



SCHEME 7.45 Enzymatic resolution of mexiletine.



SCHEME 7.46 Resolution of (±)-**154**, precursor of labetalol.

aqueous medium using phenylacetamide (**155**) as the acyl donor [100]. From this process, phenylacetamide (R)-**156** was obtained with ee = 96% (*c* = 45%, *E* = 120).

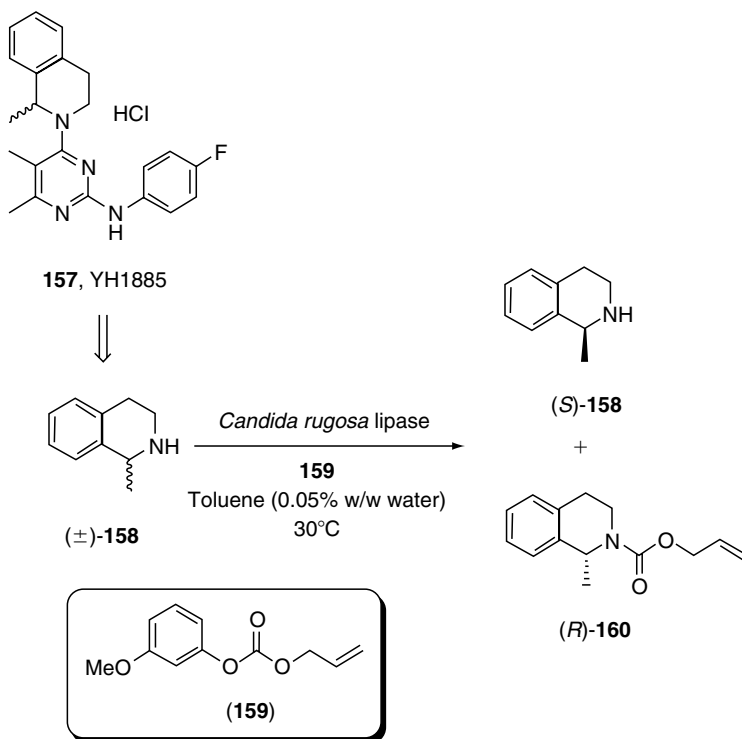
7.4.1.7 YH1885 (**157**)

The pyrimidinic compound **157** has showed utility for the treatment of gastroesophageal reflux disease and duodenal ulcers. Synthesis of racemic **157** has been carried out from (±)-1-methyl-1,2,3,4-tetrahydroisoquinoline (Scheme 7.47) [101], but recently this secondary amine has been efficiently resolved by lipase-catalyzed alkoxycarbonylation reaction. Thus, the treatment of (±)-**158** with allyl *m*-methoxyphenyl carbonate (**159**) in toluene containing 0.05% w/w water in the presence of chiroCLEC-CR, a cross-linked enzyme crystal of CRL, afforded (S)-**158** (yield = 46%; ee = 99.6%) and (R)-**160** (yield = 47%; ee = 98.4%) after 8 h of reaction [102].

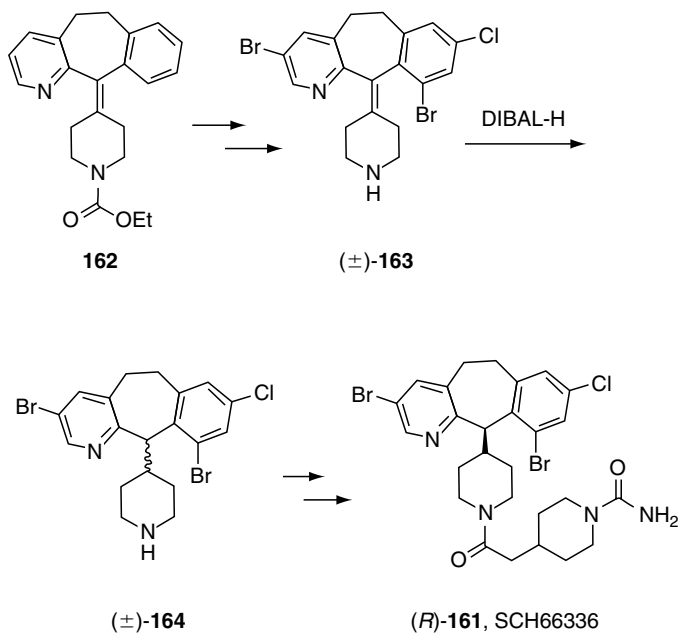
7.4.1.8 SCH66336 (**161**)

SCH66336 [(R)-**161**] is a selective, nonpeptide, nonsulfhydryl farnesyl protein transfer inhibitor that is currently undergoing phase II clinical trials for the treatment of solid tumors [103]. In the synthesis of (R)-**161** from compound **162** (Scheme 7.48), two racemic intermediates (±)-**163** and (±)-**164** are required to be resolved by enzymatic processes. Compound **163** does not contain chiral center, but it exists as a pair of enantiomers due to atropisomerism about the exocyclic double bond. Both (±)-**163** and (±)-**164** have been efficiently resolved by enzymatic aminolysis reactions [104].

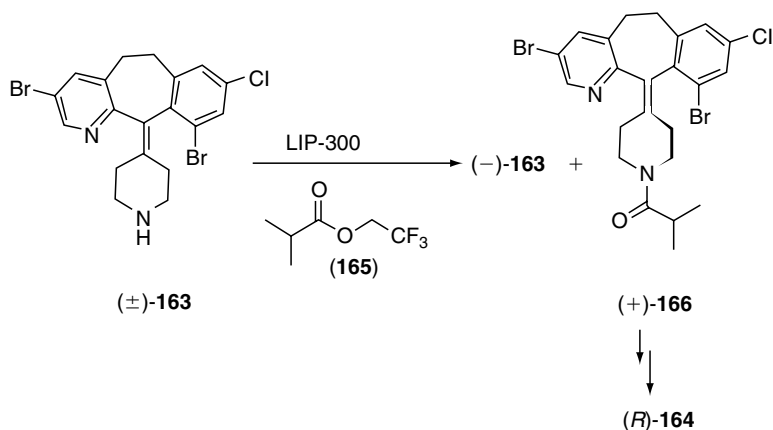
After an exhaustive screening of reaction conditions (233 commercially available enzymes, 12 organic solvents, and a wide variety of esters and carbonates as acylating agents), best results for the resolution of (±)-**163** were obtained with Toyobo LIP-300 (a lipoprotein lipase from *Pseudomonas aeruginosa*), TBME as solvent, and 2,2,2-trifluoroethyl isobutyrate (**165**) as acyl donor (Scheme 7.49). The acylation of (±)-**163** yielded (+)-**166** (ee = 97%) and



SCHEME 7.47 Resolution of (±)-**158**, precursor of YH1885.



SCHEME 7.48 Synthesis of (R)-**161**.



SCHEME 7.49 Resolution of $(\pm)\text{-163}$, a precursor of SCH66336.

$(-)\text{-163}$ (ee = 96.3%) after 24 h of reaction ($E > 200$). Conventional hydrolysis of $(+)\text{-166}$ afforded $(+)\text{-163}$, which produces the desired $(R)\text{-}(+)\text{-164}$ on reduction. The unwanted $(-)\text{-163}$ can be racemized by refluxing in di(ethyleneglycol) dibutyl ether and again subjected to enzymatic resolution. Following this strategy, 65% overall yield of $(+)\text{-163}$ (ee = 98.7%) was obtained after three rounds of enzymatic resolution.

Wide variety of reaction conditions were evaluated for the resolution of $(\pm)\text{-164}$, the best results were obtained with the same conditions that were used for $(\pm)\text{-163}$. In this case, the reaction also proceeded with high enantioselectivity ($E > 100$) and either $(S)\text{-164}$ or the produced $(R)\text{-isobutyramide}$ was obtained with very high ee (98.5% and 94.3%, respectively).

7.4.2 RESOLUTION OF 1,2-DIAMINES

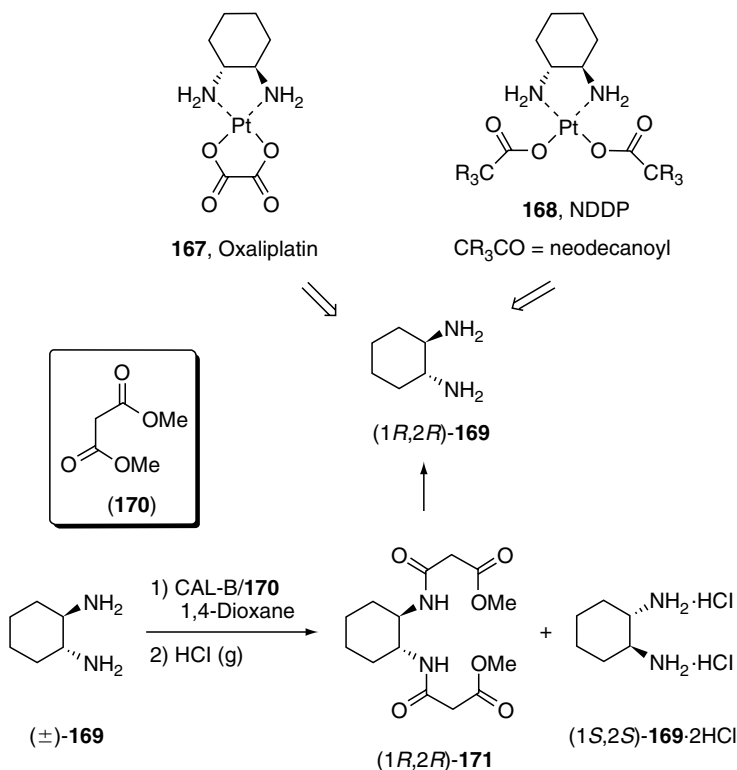
7.4.2.1 *trans*-Cyclohexane-1,2-Diamine Derivatives: Oxaliplatin (167) and NDDP (168)

The cyclohexane-1,2-diamine unit can be found in various compounds displaying a broad spectrum of biological activity [105,106]. Oxaliplatin, [*trans*-(1*R*,2*R*)-cyclohexane-1,2-diamine]oxalatoplatinum(II), has recently been approved for combination chemotherapy of metastatic colorectal cancer. Oxaliplatin (Scheme 7.50) is significantly more active than its *trans*-(1*S*,2*S*) isomer and the racemic mixture. In addition, another platinum complex derived from *trans*-(1*R*,2*R*)-cyclohexane-1,2-diamine, NDDP (168), is also an antitumoral agent currently in phase II clinical trials.

An excellent method to prepare both enantiomers of *trans*-cyclohexane-1,2-diamine (169) with very high ee consisted the sequential KR of $(\pm)\text{-169}$ using CAL-B as catalyst and dimethyl malonate (170) as acyl donor (Scheme 7.50) [107]. The enzyme catalyzed the monoacylation of the diamine with $E = 45$, and the subsequent acylation of the resulting enantioenriched (1*R*,2*R*)-monoamide ($E = 68$) afforded the diamide (1*R*,2*R*)-171 with >99% ee and 45% yield. Enantiopure diamine (1*S*,2*S*)-169 was obtained when the reaction was performed with a slightly excess acyl donor.

7.4.2.2 U-50,488 (172)

Compound U-50,488 (172) and other structural analogs have been reported to be highly selective κ -opioid agonists, free from the adverse side effects of μ agonists like morphine. The majority of these pharmacologically active compounds have similar configuration at the



SCHEME 7.50 Enzymatic preparation of (1R,2R)-169, precursor of the antitumoral agents 167 and 168.

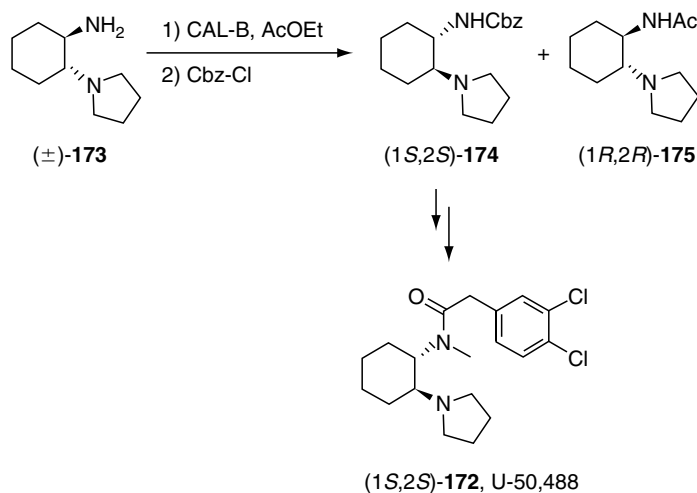
stereogenic centers. Thus, (1S,2S)-(–)-172 (Scheme 7.51) exhibits greater κ agonist activity than its enantiomer, and the *cis* diastereomer of U-50,488 has practically no affinity for κ receptors [108].

(1S,2S)-172 has been obtained by a chemoenzymatic route starting from the inexpensive cyclohexene oxide [109]. The key steps involved in this route were the stereospecific synthesis of (±)-*trans*-2-(pyrrolidin-1-yl)cyclohexanamine 173 and its subsequent enzymatic resolution by CAL-B catalyzed aminolysis of ethyl acetate. The enzymatic process gave very high enantioselectivity ($E = 170$), CAL-B preferentially catalyzed the acetylation of the (1R,2R) enantiomer of 173. To facilitate the isolation of the amide (1R,2R)-175 (ee = 94%) and the unreacted diamine (1S,2S)-173 (ee = 99%), the reaction mixture was treated with benzylchloroformate. Thus, (1S,2S)-173 was transformed into the benzylcarbamate (1S,2S)-174 and the mixture formed by 174 and 175 was easily separated by flash chromatography. Finally, carbamate (1S,2S)-174 was submitted to reduction with lithium aluminum hydride (LAH) and subsequent amidation with 3,4-dichlorophenylacetyl chloride to yield the analgesic (1S,2S)-172.

7.5 RESOLUTION OF ESTERS BY AMINOLYSIS OR AMMONOLYSIS

7.5.1 IBUPROFEN

A derivative of the anti-inflammatory ibuprofen, its 2-chloroethyl ester (S)-176, has been obtained with ee = 96% by CAL-B catalyzed ammonolysis of (±)-176 (Scheme 7.52) [110]. Reaction was performed in *tert*-butyl alcohol as solvent and ammonia was bubbled through



SCHEME 7.51 Chemoenzymatic synthesis of U-50,488.

the solution. Under these conditions, the enzyme catalyzed the ammonolysis of (*R*)-**176** with moderate enantioselectivity ($E = 28$).

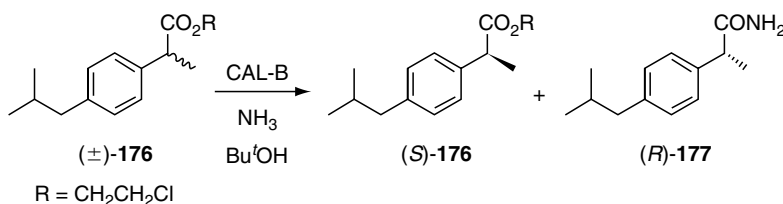
7.5.2 3-PYRROLIDINOL DERIVATIVES **178–181**

(*S*)-3-Pyrrolidinol [(*S*)-**182**] is a precursor of a variety of compounds with activity as κ -receptor agonists. [Scheme 7.53](#) demonstrates some of these compounds: **178** [111], **179** [112], **180** [113], and **181** [114].

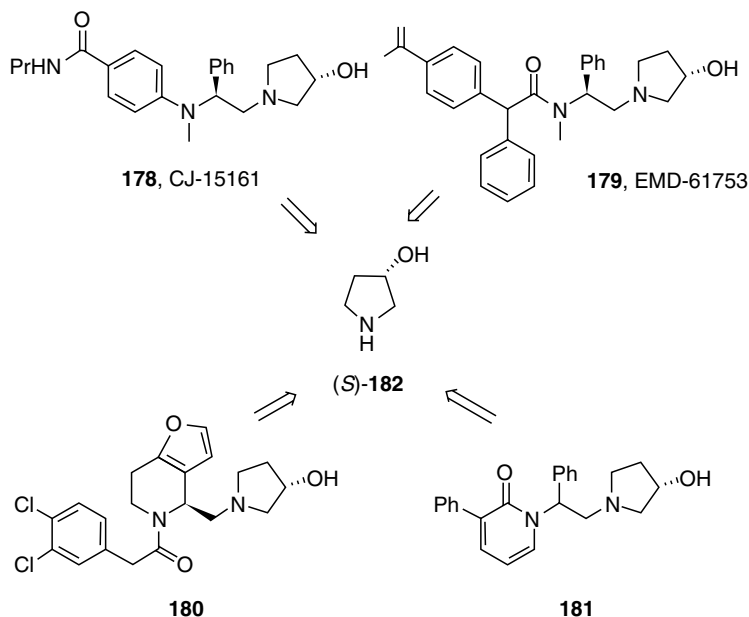
A chemoenzymatic synthesis of (*S*)-**182** has been carried out from ethyl (\pm)-4-chloro-3-hydroxybutanoate (**183**), the key step being the resolution of (\pm)-**183** by ammonolysis reaction catalyzed by CAL-B in 1,4-dioxane as a solvent. CAL-B catalyzed the ammonolysis of (*S*)-**183** with moderate enantioselectivity, the corresponding amide (*S*)-**184** being isolated with 93% ee after 1.5 h reaction ([Scheme 7.54](#)). The amide (*S*)-**184** was easily transformed into (*S*)-**182** (87% of overall yield) [115].

7.5.3 (*R*)-GABOB (**185**)

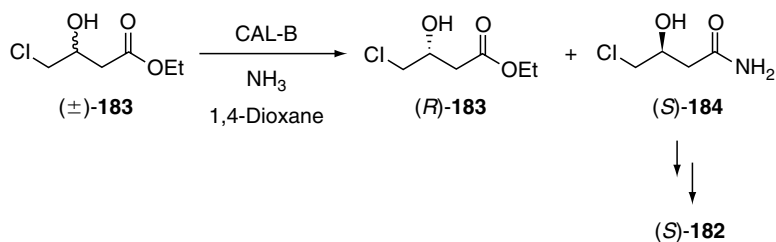
(*R*)-4-Amino-3-hydroxybutanoic acid [(*R*)-GABOB] is a compound of great importance because of its biological function as a neuromodulator in the mammalian central nervous system. Moreover, (*R*)-GABOB (**185**) is a precursor of (*R*)-carnitine (**186**, vitamin BT), a therapeutic agent for the treatment of myocardial ischemia. (*R*)-GABOB has been synthesized by a chemoenzymatic route involving the asymmetric ammonolysis of dimethyl



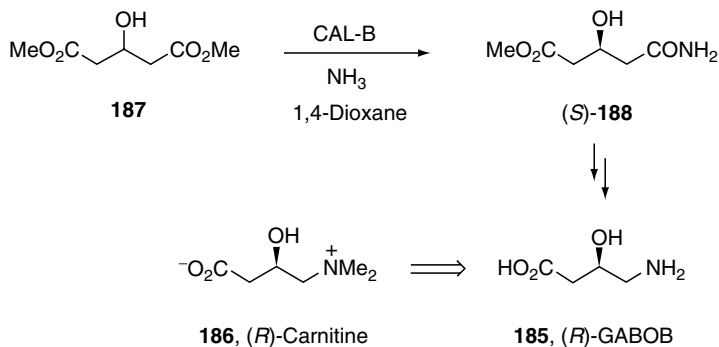
SCHEME 7.52 Resolution of (\pm)-**176**.



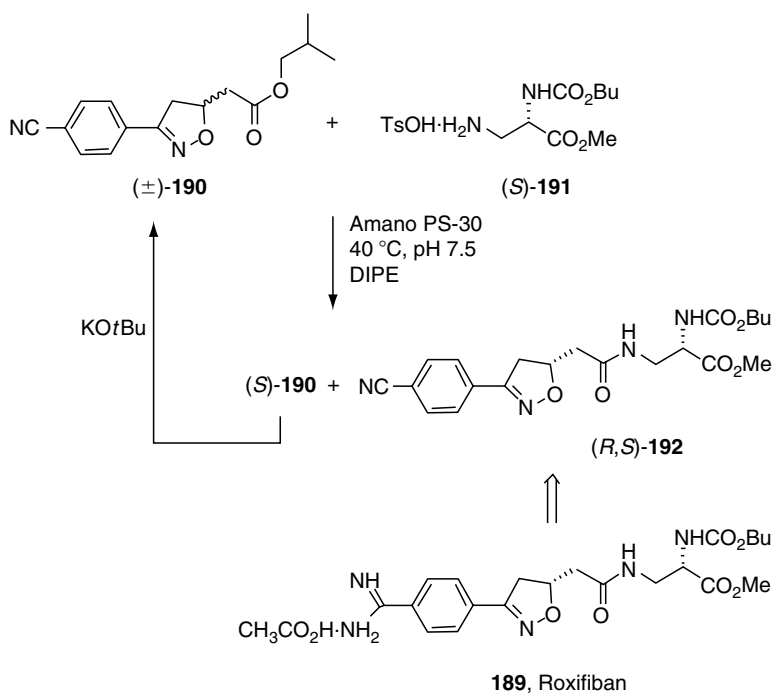
SCHEME 7.53 3-Pyrrolidinol derivatives.



SCHEME 7.54 Chemoenzymatic synthesis of (S)-3-pyrrolidinol.



SCHEME 7.55 Chemoenzymatic synthesis of (R)-GABOB.



SCHEME 7.56 Enzymatic preparation of (R,S) -**192**, precursor of roxifiban.

3-hydroxyglutarate (**187**) catalyzed by CAL-B (Scheme 7.55). The reaction was carried out with ammonia saturated 1,4-dioxane, the enzyme catalyzing the transformation of the *pro-R* group of the ester, affording the enantiopure monoamide (S) -**188**. From this monoamide, (R) -GABOB was obtained by four sequential synthetic steps [116].

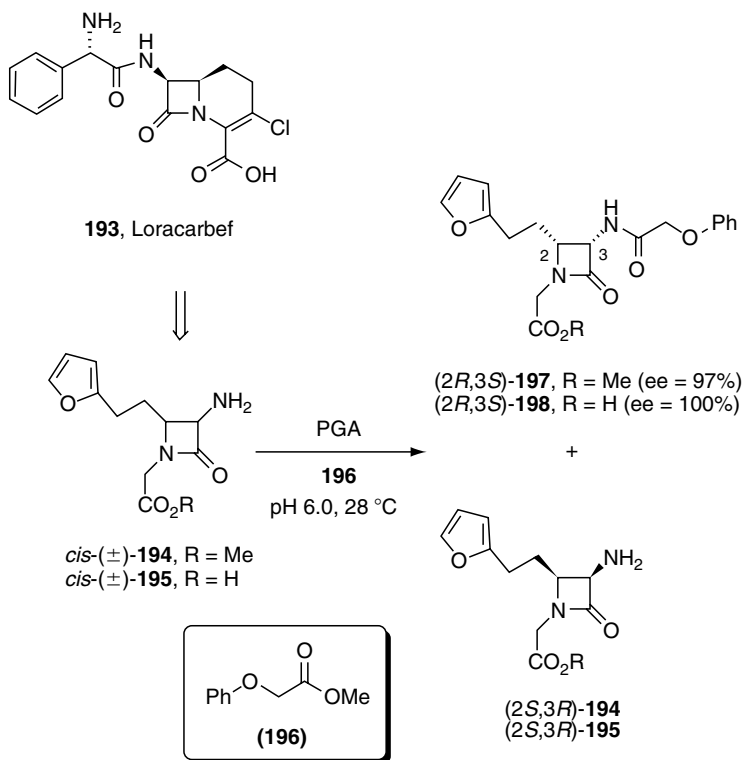
7.5.4 ROXIFIBAN (**189**)

Roxifiban is a nonpeptide platelet glycoprotein IIb/IIIa antagonist with antithrombotic activity. A precursor of roxifiban is the optically active isoxazol derivative (R,S) -**192** [117], which has been prepared by aminolysis of the racemic isobutyl 2-isoxazolyacetate ((\pm) -**190**) using a pretreated lipase Amano PS-30 [118]. As shown in Scheme 7.56, nucleophile was the enantiopure diaminoester derivative (S) -**191** that was used as its *p*-toluenesulfonate salt because the free amine was unstable. When enzymatic reaction was carried out in DIPE, the formation of (R,S) -**192** occurs with high diastereoselectivity [diastereomeric excess (de) of 86%]. Although CAL-B also catalyzed this aminolysis reaction yielding (R,S) -**192** with even higher de (92%), the competitive hydrolysis of the ester ((\pm) -**190**) took place at greater rate than with lipase PS-30. The unreacted (S) -**190** can be recovered, racemized by treatment with KOtBu , and recycled in a next enzymatic aminolysis reaction.

7.6 RESOLUTION OF AMINO ACID DERIVATIVES BY *N*-ACYLATION REACTIONS

7.6.1 LORACARBEF (**193**)

Loracarbef is an orally absorbable synthetic β -lactam antibiotic that is characterized by an enhanced chemical stability. In a synthesis of **193** the racemic azetidinone *cis*-(\pm)-**194** was used as key intermediate, the $(2R,3S)$ -isomer being used in subsequent chemistry to the



SCHEME 7.57 Resolution of azetidinones **194** and **195**, precursors of loracarbef.

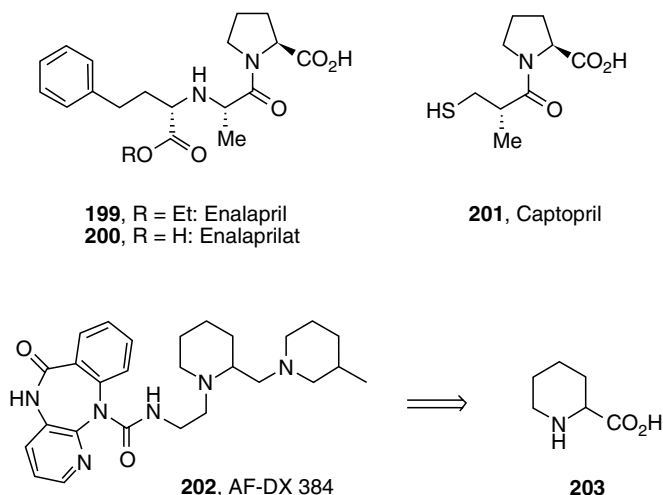
antibiotic (Scheme 7.57). The required (2*R*,3*S*)-azetidinone was obtained by the enzymatic resolution of either *cis*-(±)-**194** or *cis*-(±)-**195**, using immobilized penicillin G amidase (PGA) from *E. coli*. [119]. Reactions were carried out in water, at pH 6.0, using methyl phenylacetate and methyl phenoxyacetate (**196**) as acyl donors. With both acyl donors, the enzyme catalyzed the formation of the amide with the required (2*R*,3*S*) configuration, with very high ee (≥96% starting from **194** and 100% starting from **195**). Scheme 7.57 demonstrates the results obtained with methyl phenoxyacetate (**196**).

The loracarbef also contains in its structure a L-phenylglycine unit. This α-amino acid can be obtained by enzymatic acylation of the corresponding racemic phenylglycine methyl ester using the PGA and methyl 4-hydroxyphenylacetate as the acyl donor in toluene or dichloromethane. The corresponding 4-hydroxyphenylacetamide derived from (*S*)-phenylglycine was obtained with a very high enantioselectivity [120].

7.6.2 PROLINE AND PIPECOLIC ACID DERIVATIVES 199–202

L-Proline and L-pipecolic acid have properties as anticonvulsants and they have been used as starting materials of many pharmacologically active compounds [121]. For instance, L-proline is a constituent of the antihypertensive enalapril (**199**), enalaprilat (**200**), and captopril (**201**) (Scheme 7.58). In addition, both enantiomers of pipecolic acid (**203**) have been used to obtain AF-DX 384 (**202**), a selective antagonist of muscarinic M₂ receptors, the (*R*)-isomer exhibiting higher affinity than (*S*)-enantiomer [122].

An efficient approach to obtain these cyclic α-amino acids with high enantiomeric purity has been demonstrated by the resolution of their corresponding racemic methyl esters



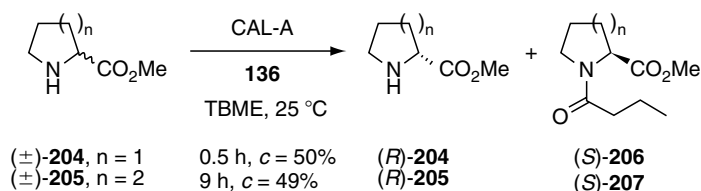
SCHEME 7.58 L-Proline and pipecolic acid derivatives.

(\pm)-**204** and (\pm)-**205** by aminolysis of 2,2,2-trifluoroethyl butanoate (**136**) using CAL-A (immobilized on celite) as catalyst and TBME as solvent. In both cases, the enzyme was very efficient in catalyzing the acylation of the secondary amino group (Scheme 7.59) leading to the formation of the butanamides (*S*)-**206** and (*S*)-**207** with very high enantioselectivities ($E > 100$) [17,123].

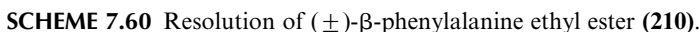
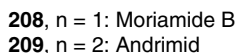
By an adequate selection of the reaction conditions the DKR of these substrates was achieved. Thus, using vinyl butanoate as the acyl donor and triethylamine as an additive, (*S*)-**206** and (*S*)-**207** were obtained with very high ee (97%) and yields (90% and 70%, respectively) [17]. The use of vinyl butanoate allows the release of acetaldehyde, which *in situ* racemizes the remaining (*R*)-**204** and (*R*)-**205**.

7.6.3 MOIRAMIDE B (**208**) AND ANDRIMID (**209**)

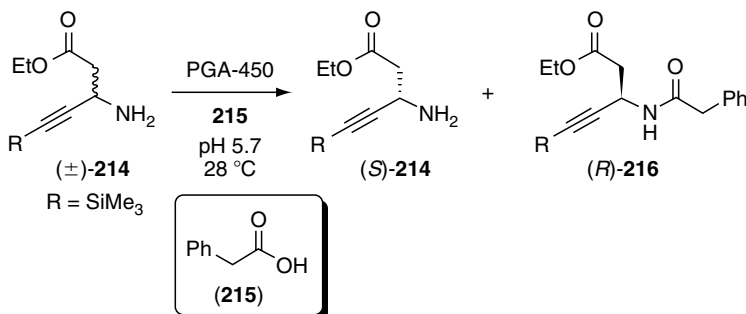
The pseudopeptide natural products moiramide B and andrimid represent a new class of antibiotics that target bacterial fatty acid biosynthesis [124]. One of the starting materials for the synthesis of **208** and **209** is the optically active D- β -phenylalanine or some of its derivatives [125]. Similar to the resolution of proline and pipecolic acid derivatives, CAL-A catalyzed the resolution of racemic β -phenylalanine ethyl ester [(\pm)-**210**] in DIPE as solvent. After 1 h of reaction with 2,2,2-trifluoroethyl butanoate (**136**), the unreacted (*R*)-**210** and the butanamide (*S*)-**211** produced by enzymatic reaction were isolated in 90% ee and 98% ee, respectively (Scheme 7.60) [126].



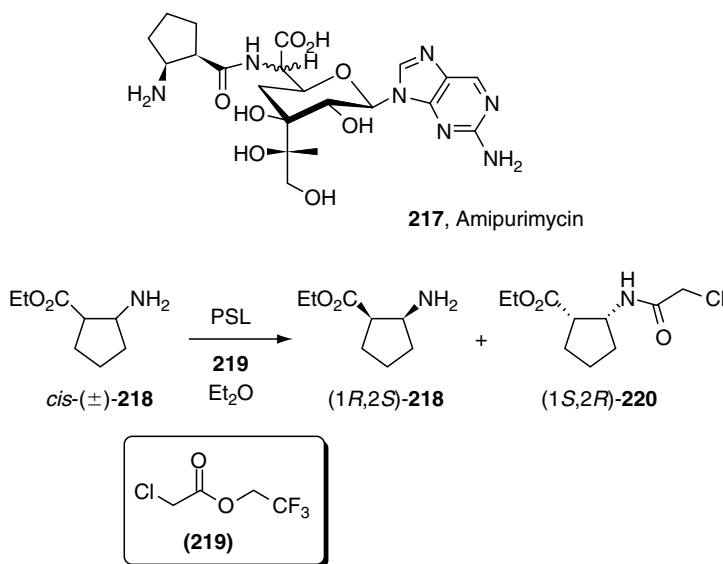
SCHEME 7.59 Resolution of (\pm)-**204** and (\pm)-**205**.



The optically active β -amino ester (*S*)-**214** (Scheme 7.61) has been used in the syntheses of xenilofiban (**212**) [127] and its analogous FR-184764 (**213**) [128]. The resolution of (\pm)-**214** has been efficiently achieved by enzymatic acylation using phenylacetic acid (**215**) as acyl donor and an immobilized PGA-450 as catalyst [129]. The reaction was carried out at pH 5.7 and 28°C. Under these conditions, the enzyme showed a very high enantioselectivity and both the phenylacetamide (*R*)-**216** and the unreacted β -amino ester (*S*)-**214** were isolated with



© 2006 by Taylor & Francis Group, LLC.



SCHEME 7.62 Resolution of (±)-**218**.

>99% ee and 98% ee, respectively. This bioconversion was scaled to resolve 6.1 kg of amine in three runs, from which the required (*S*)-**214** was isolated in 96 to 98% ee and 43 to 46% yield.

7.6.5 AMIPURIMYCIN (**217**)

In addition to the already described pharmacological properties of (1*R*,2*S*)-2-aminocyclopentane carboxylic acid (**77**: cispentacin, [Scheme 7.24](#)), this β-amino acid is also a component of the antibiotic amipurimycin (**217**, [Scheme 7.62](#)). An alternative method described in [Scheme 7.24](#) for the preparation of enantiomerically pure cispentancin consists of the resolution of its racemic ethyl ester (±)-**218** by *N*-acylation reaction catalyzed by lipases [130]. Using a pretreated PS lipase, 2,2,2-trifluoroethyl acetate as acyl donor, and diethyl ether as solvent, acetylation of the (1*S*,2*R*) enantiomer of the β-amino ester occurs with very high enantioselectivity (*E* >100), giving 49% of conversion after 1 h reaction. When a more activated ester such as 2,2,2-trifluoroethyl chloroacetate (**219**) was used, the reaction rate increased, 51% of conversion being achieved only after 5 min of the reaction. From this process, (1*R*,2*S*)-**218** and chloroacetamide (1*S*,2*R*)-**220** were isolated with 99.7% ee and 95% ee, respectively.

7.7 CONCLUDING REMARKS

The use of biocatalysis has become a conventional tool for organic and bioorganic chemists. Their utility in carrying out very selective transformations under mild reaction conditions makes them a very attractive catalyst to perform some transformations in a synthetic route. Pharmaceutical companies are using biocatalytic processes for the preparation of chiral drugs because they offer enormous advantages, such as their environmental friendly properties and the low cost of the processes. In addition, we strongly believe that the genetic engineering techniques will continue to play a major role in the future research in biocatalysis and will open up new possibilities to carry out industrial processes in several sectors. In this chapter, we have shown how enzymatic reactions using hydrolases, mainly acylation of alcohols and amines, have provided potential application for the preparation of chiral pharmaceuticals during the past few years.

REFERENCES

1. Zaks, A., Industrial biocatalysis, *Curr. Opin. Chem. Biol.*, 5(2), 130–136, 2001.
2. Roberts, S.M., Biocatalysis in synthetic organic chemistry, *Tetrahedron*, 60(3), 499–500, 2004.
3. Thomas, S.M., DiCosimo, R., and Nagarajan, V., Biocatalysis: applications and potentials for the chemical industry, *Trends Biotechnol.*, 20(6), 238–242, 2002.
4. Federsel, H.J., Drug chirality-scale-up manufacturing and control, *CHEMTECH*, 23(12), 24–33, 1993.
5. Gotor, V., Biocatalysis applied to the preparation of pharmaceuticals, *Org. Proc. Res. Dev.*, 6(4), 420–426, 2002.
6. Straathof, A.J.J., Panke, S., and Schmid, A., The production of fine chemicals by biotransformations, *Curr. Opin. Biotechnol.*, 13(6), 548–556, 2002.
7. Huisman, G.W. and Gray, D., Towards novel processes for the fine chemical and pharmaceutical industries, *Curr. Opin. Biotechnol.*, 13(4), 352–358, 2002.
8. Patel, R.N., Microbial/enzymatic synthesis of chiral intermediates for pharmaceuticals, *Enzyme Microb. Tech.*, 31(6), 804–826, 2002.
9. Patel, R.N., Microbial/enzymatic synthesis of chiral pharmaceutical intermediates, *Curr. Opin. Drug. Disc. Dev.*, 6(6), 902–920, 2003.
10. Reetz, M.T., Lipases as practical biocatalysts, *Curr. Opin. Chem. Biol.*, 6(2), 145–150, 2002.
11. Ghanem, A. and Aboul-Enin, H.Y., Lipase mediated chiral resolution of racemates in organic solvents, *Tetrahedron Asymmetry*, 15(21), 3331–3351, 2004.
12. Alfonso, I. and Gotor, V., Biocatalytic and biomimetic aminolysis reactions: useful tools for selective transformations on polifunctional substrates, *Chem. Soc. Rev.*, 33(4), 201–209, 2004.
13. Kato, K., Goug, Y., Saito, T., et al., Enzymatic resolution of 2,2,2-trifluoro-1-arylethylamine derivatives by *Pseudomonas fluorescens* lipase in organic solvents, *J. Mol. Catal., B Enzym.*, 30(2), 61–68, 2004.
14. Faber, K., Non-sequential processes for the transformation of a racemate into a single stereoisomer product. Proposal for stereochemical classification, *Chem. Eur. J.*, 7(23), 5005–5010, 2001.
15. Pellissier, H., Dynamic kinetic resolution, *Tetrahedron*, 59(42), 8291–8327, 2003.
16. Pàmies, O. and Bäckvall, J.E., Combination of enzymes and metal catalysis. A powerful approach in asymmetric catalysis, *Chem. Rev.*, 103(8), 3247–3262, 2003.
17. Liljeblad, A., Kiviniemi, A., and Kanerva, L.T., Aldehyde-based racemization in the dynamic kinetic resolution of *N*-heterocyclic α -aminoesters using *Candida antarctica* lipase A, *Tetrahedron*, 60(3), 671–677, 2004.
18. Vedejs, E. and Chen, X., Parallel kinetic resolution, *J. Am. Chem. Soc.*, 119(10), 2584–2585, 1997.
19. Dehli, J.R. and Gotor, V., Parallel kinetic resolution of racemic mixtures: a new strategy for the preparation of enantiopure compounds? *Chem. Soc. Rev.*, 31(6), 365–370, 2002.
20. Wills, M.C.J., Enantioselective desymmetrization, *J. Chem. Soc. Perkin Trans.*, 1(13), 1765–1784, 1999.
21. Schoffers, E., Gelebiowski, A., and Johnson C.R., Enantioselective synthesis through enzymatic asymmetrization, *Tetrahedron*, 52(11), 3769–3826, 1996.
22. García-Urdiales, E., Alfonso, I., and Gotor, V., Enantioselective desymmetrization in organic synthesis, *Chem. Rev.*, 105(1), 313–354, 2005.
23. Gotor, V., Pharmaceuticals through enzymatic transesterification and aminolysis reactions, *Bio-catal. Biotransformation*, 18(2), 87–103, 2000.
24. Pesti, J.A. and DiCosimo, R., Recent progress in enzymatic resolution and desymmetrization of pharmaceuticals and their intermediates, *Curr. Opin. Drug. Discov. Devel.*, 6(6), 884–901, 2003.
25. Margolin, A.L., Enzymes in the synthesis of chiral drugs, *Enzyme Microb. Technol.*, 15(4), 266–280, 1993.
26. Schulze, B. and Wubbolts, M.G., Biocatalysis for industrial production of fine chemicals, *Curr. Opin. Biotechnol.*, 10(6), 609–615, 1999.
27. Patel, R.N. (Ed.), *Stereoselective Biocatalysis*, Marcel Dekker, New York, 2000, pp. 87–130.
28. Patel, R.N., Enzymatic synthesis of chiral intermediates for drug development, *Adv. Synth. Catal.*, 343(6–7), 527–546, 2001.

29. Patel, R.N., Biocatalytic synthesis of intermediates for the synthesis of chiral drugs substances, *Curr. Opin. Biotechnol.*, 12(6), 587–604, 2001.
30. Roberts, S.M., Preparative biotransformations, *J. Chem. Soc. Perkin Trans.*, 1, 1475–1499, 2001.
31. Jaeger, K.-E. and Eggert, T., Lipases for biotechnology, *Curr. Opin. Biotechnol.*, 13(4), 390–397, 2002.
32. Laumen, K., Kittelmann, M., and Ghisalba, O., Chemo-enzymatic approaches for the creation of novel chiral building blocks and reagents for pharmaceutical applications, *J. Mol. Catal., B Enzym.*, 19–20, 55–66, 2002.
33. Patel, R.N., Enzymatic preparation of chiral pharmaceutical intermediates by lipases, *Lipid Biotechnol.*, 527–561, 2002.
34. Park, H.G., Do, J.H., and Chang, N.C., Regioselective enzymatic acylation of multi-hydroxyl compounds in organic synthesis, *Biotechnol. Bioprocess Eng.*, 8(1), 1–8, 2003.
35. Im, D.S., Cheong, C.S., Lee, S.H., et al., Chemo-enzymatic synthesis of (*R*)-(+)-aminoglutethimide by kinetic resolution of (\pm)-4-cyano-4-phenyl-1-hexanol, *J. Mol. Catal., B Enzym.*, 26(3–6), 185–191, 2003.
36. Ferraboschi, P., Pecora, F., Reza-Elahi, S., et al., Chemoenzymatic syntheses of (25*R*)- and (25*S*)-25-hydroxy-27-nor-cholesterol, a steroid bearing a secondary hydroxy group in the side chain, *Tetrahedron Asymmetry*, 10(13), 2497–2500, 1999.
37. Hiroya, K., Zhang, H., and Ogasawara, K., Preparation of the synthetic equivalents of chiral cyclohexadienone and cycloheptadienone. The enantio- and diastereo-controlled synthesis of (–)-clavularin B, *Synlett*, (5), 529–532, 1999.
38. Mehta, G. and Reddy, D.S., Enzymatic resolution of dioxygenated dicyclopentadienes: enantio-pure hydrindanes, hydroisquinolones, diquinanes and application to a synthesis of (+)-coronafacic acid, *Tetrahedron Lett.*, 40(5), 991–994, 1999.
39. Morishita, K., Kamezawa, M., Ohtani, T., et al., Chemoenzymic synthesis of (+)-docosa-4,15-dien-1-yn-3-ol, a component of the marine sponge *Cribrochalina vasculum*, confirmation of the structure and absolute configuration of the acetylenic alcohol, by lipase-catalyzed biotransformations, *J. Chem. Soc. Perkin Trans.*, 1(4), 513–518, 1999.
40. Frigola, J., Berrocal, J.M., Cuberes, M.R., et al., Procédé de séparation de carbinols, French Patent 235497 D16049-FA, 1995.
41. Zhu, B. and Panek, J.S., Methodology based on chiral silanes in the synthesis of polypropionate-derived natural products—total synthesis of epothiolone A, *Eur. J. Org. Chem.*, (9), 1701–1714, 2001.
42. Liu, H.-L., Helge, B., and Anthonsen, T., Chemoenzymatic synthesis of the non-tricyclic antidepressants fluoxetine, tomoxetine and nisoxetine, *J. Chem. Soc. Perkin Trans.*, 1(11), 1767–1769, 2000.
43. Kamal, A., Khanna, G.B.R., and Ramu, R., Chemoenzymatic synthesis of both enantiomers of fluoxetine, tomoxetine and nisoxetine: lipase catalyzed resolution of 3-aryl-3-hydroxypropanenitriles, *Tetrahedron Asymmetry*, 13(18), 2039–2051, 2002.
44. Campos, F., Bosch, M.P., and Guerrero, A., An efficient enantioselective synthesis of (*R,R*)-formoterol, a potent bronchodilator, using lipases, *Tetrahedron Asymmetry*, 11(13), 2705–2717, 2000.
45. Patel, R.N., McNamee, C.G., and Szarka, L.J., Enantioselective enzymatic acetylation of racemic 4-4- α ,6- β (*E*)-6,4,4-bis(4-fluorophenyl)-3-(1-methyl-1-*H*-tetrazol-5-yl)-1,3-butadienyl-tetrahydro-4-hydroxy-2H-pyran-2-one, *Appl. Microbiol. Biotechnol.*, 38(1), 56–60, 1992.
46. Nagai, H., Shizawa, T., and Achiwa, K., Convenient syntheses of optically active β -lactams by enzymic resolution, *Chem. Pharm. Bull.*, 41(11), 1933–1938, 1993.
47. Sankaranarayanan, S., Sharma, A., Kulkarni, B.A., et al., Preparation of the versatile chiron, (*R*)- and (*S*)-12-(tetrahydropyranyloxy)-3-methyldodecanol: application to the syntheses of methyl branched insect pheromones, *J. Org. Chem.*, 60(12), 4251–4253, 1995.
48. Kulkarni B.A., Sankaranarayanan S., Subbaraman A.S., et al., Synthesis of racemic and each enantiomer of 3-methylnonacosanol, a new plant growth regulator from *Lowsonia inermis*, *Tetrahedron Asymmetry*, 10(8), 1571–1577, 1999.
49. Kapoor, M., Anand, N., Ahmad, K., et al., Synthesis of β -adrenergic blockers (*R*)-(–)-nifenalol and (*S*)-(+)-sotalol via a highly resolution of a bromohydrin precursor, *Tetrahedron Asymmetry*, 16(3), 717–725, 2005.

50. Ebiiike, H., Ozawa, Y., Achiwa, K., et al., Enzyme-catalysed synthesis of biologically active (S)-nilvadipine, *Heterocycles*, 35(2), 603–606, 1993.
51. Kitano, K., Matsubara, J., Ohtani, T., et al., An efficient synthesis of optically active metabolites of platelet adhesion inhibitor OPC-29030 by lipase-catalyzed enantioselective transesterification, *Tetrahedron Lett.*, 40(28), 5235–5238, 1999.
52. Pàmies, O. and Bäckvall, J.-E., Dynamic kinetic resolution of β -azido alcohols. An efficient route to chiral aziridines and β -aminoalcohols, *J. Org. Chem.*, 66(11), 4022–4025, 2001.
53. Wang, Y.-F., Chen, S.-T., Liu, K.-C., et al., Lipase-catalyzed irreversible transesterification using enol esters: resolution of cyanohydrins and syntheses of ethyl (*R*)-2-hydroxy-4-phenylbutyrate and (*S*)-propanolol, *Tetrahedron Lett.*, 30(15), 1917–1920, 1989.
54. Bevinakatti, H.S. and Banerji, A.A., Practical chemoenzymatic synthesis of both enantiomers of propanolol, *J. Org. Chem.*, 56(18), 5372–5375, 1991.
55. Cregge, R.J., Wagner, E.R., Freedman, J., et al., Lipase-catalyzed transesterification in the synthesis of a new chiral unlabeled and carbon-14 labeled serotonin uptake inhibitor, *J. Org. Chem.*, 55(14), 4237–4238, 1990.
56. Carr, A.A., Nieduzak, T.R., and Miller, F.P., 1,4-Disubstituted piperidine derivatives and their preparation, pharmaceutical compositions, and use in analgesics and muscle relaxants, EP 317997, CAN 111:232581, 1989.
57. Sih, J.C., Application of immobilized lipase in production of camptosar (CPT-11), *J. Am. Oil Chem. Soc.*, 73(11), 1377–1378, 1996.
58. Theil, F. and Ballschuh, S., Chemoenzymatic synthesis of both enantiomers of cispentacin, *Tetrahedron Asymmetry*, 7(12), 3565–3572, 1996.
59. Gais, H.-J., Griebel, C., and Buschmann, H., Enzymatic resolution of analgesics: δ -hydroxytramadol, ϵ -tramadol and *O*-desmethytramadol, *Tetrahedron Asymmetry*, 11(4), 917–928, 2000.
60. Shimada, K., Kaburagi, Y., and Fukuyama, T., Total synthesis of leustroduscin B., *J. Am. Chem. Soc.*, 125(14), 4048–4049, 2003.
61. Terao, Y., Murata, M., Achiwa, K., et al., Highly efficient lipase-catalyzed asymmetric synthesis of chiral glycerol derivatives leading to practical synthesis of *s*-propanolol, *Tetrahedron Lett.*, 29(40), 5173–5176, 1988.
62. Saksena, A.K., Girijavallabhan, V.M., Lovey, R.G., et al., Highly stereoselective access to novel 2,2,4-trisubstituted tetrahydrofurans by halocyclization-practical chemoenzymatic synthesis of Sch-51048, a broad spectrum orally active antifungal agent, *Tetrahedron Lett.*, 36(11), 1787–1790, 1995.
63. Bovara, R., Carrea, G., Ferrara, L., et al., Resolution of (\pm)-*trans*-sobrerol by lipase PS-catalyzed transesterification and effects of organic solvents on enantioselectivity, *Tetrahedron Asymmetry*, 2(9), 931–938, 1991.
64. Akai, S., Tsujino, T., Naka, T., et al., Lipase-catalysed enantioselective desymmetrization of prochiral 3,3-bis(hydroxymethyl)oxindoles, *Tetrahedron Lett.*, 42(41), 7315–7317, 2001.
65. Akai, S., Tsujino, T., Akiyama, E., et al., Enantiodivergent preparation of optically active oxindoles having a stereogenic quaternary carbon center at the C3 position via the lipase-catalyzed desymmetrization protocol: effective use of 2-furoates for either enzymatic esterification or hydrolysis, *J. Org. Chem.*, 69(7), 2478–2486, 2004.
66. Colombo, D., Compostella, F., Ronchetti, F., et al., Chemoenzymatic synthesis and antitumor promoting activity of 6'- and 3-esters of 2-*O*- β -D-glucosyglycerol, *Bioorg. Med. Chem.*, 7(9), 1867–1871, 1999.
67. Laumen, K. and Ghisalba O., Preparative-scale chemo-enzymic synthesis of optically pure D-*myo*-inositol 1-phosphate, *Biosci. Biotechnol. Biochem.*, 58(11), 2046–2049, 1994.
68. Laumen, K. and Ghisalba O., Chemo-enzymatic synthesis of both enantiomers of *myo*-inositol 1,3,4,5-tetrakisphosphate, *Biosci. Biotechnol. Biochem.*, 63(8), 1374–1377, 1999.
69. Chênevert, R., Simard, M., Bergeron, J., et al., Chemoenzymatic formal synthesis of (*S*)-(-)-phosphonotrixin, *Tetrahedron Asymmetry*, 15(12), 1889–1892, 2004.
70. Chênevert, R. and Courchesne, G., Synthesis of (*S*)- α -tocotrienol via an enzymatic desymmetrization of an achiral chroman derivative, *Tetrahedron Lett.*, 43(44), 7971–7973, 2002.

71. Chênevert, R., Ziarani, G.M., Morin, M.P., et al., Enzymatic desymmetrization of *meso cis*-2,6- and *cis*, *cis*-2,4,6-substituted piperidines. Chemoenzymatic synthesis of (5*S*,9*S*)-(+)-indolizidine 209D, *Tetrahedron Asymmetry*, 10(16), 3117–3122, 1999.
72. Mase, N., Nishi, T., Takamori, Y., et al., First synthesis of (*R*)-(-)-5-hydroxy-3-methyl-3-pyrrolin-2-one (jatropham) by lipase-catalyzed kinetic resolution, *Tetrahedron Asymmetry*, 10(23), 4469–4471, 1999.
73. Lundell, K., Katainen, E., Kiviniemi, A., et al., Enantiomers of adrenaline-type aminoalcohols by *Burkholderia cepacia* lipase-catalyzed asymmetric acylation, *Tetrahedron Asymmetry*, 15(23), 3723–3729, 2004.
74. Franceschini, N., Joy, M.S., and Kshirsagar, A., Cinacalcet HCl: a calcimimetic agent for the management of primary and secondary hyperparathyroidism, *Expert Opin. Investig. Drugs*, 12(8), 1413–1421, 2003.
75. Van Wagenen, B.C., Moe, S.T., Balandrin, M.F., et al., Preparation of 1-arylethylamines as calcium receptor ligands, US 6211244, CAN 134:280612, 2001.
76. Skupinska, K.A., McEachern, E.J., Baird, I.R., et al., Enzymatic resolution of bicyclic 1-hetero-aryl amines using *Candida antarctica* lipase B, *J. Org. Chem.*, 68(9), 3546–3551, 2003.
77. Reetz, M.T. and Dreisbach, C., Highly efficient lipase-catalyzed kinetic resolution of chiral amines, *Chimia*, 48(12), 570, 1994.
78. Reeve, C.D., Resolution of chiral amines, WO 9931264, CAN 131:43668, 1999.
79. García-Urdiales, E., Rebollo, F., and Gotor, V., Enzymatic one-pot resolution of two nucleophiles: alcohol and amine, *Tetrahedron Asymmetry*, 11(7), 1459–1463, 2000.
80. Jaeger, K.-E., Schneider, B., Rosenau, F., et al., Bacterial lipases for biotechnological applications, *J. Mol. Catal., B Enzym.*, 3(1–4), 3–12, 1997.
81. Kitaguchi, H., Fitzpatrick, P.A., Huber, J.E., et al., Enzymic resolution of racemic amines: crucial role of the solvent, *J. Am. Chem. Soc.*, 111(8), 3094–3095, 1989.
82. Margolin, A.L., Fitzpatrick, P.A., Dubin, P.L., et al., Chemoenzymic synthesis of optically active (meth)acrylic polymers, *J. Am. Chem. Soc.*, 113(12), 4693–4694, 1991.
83. Takayama, S., Moree, W.J., and Wong, C.-H., Enzymic resolution of amines and amino alcohols using pent-4-enoyl derivatives, *Tetrahedron Lett.*, 37(35), 6287–6290, 1996.
84. Gutman, A.L., Meyer, E., Kalerin, E., et al., Enzymatic resolution of racemic amines in a continuous reactor in organic solvents, *Biotechnol. Bioeng.*, 40(7), 760–767, 1992.
85. Youdim, M.B.H., Finberg, J.P.M., Levy, R., Sterling, J., Lerner, D., and Berger-Paskin, T., *R*-enantiomers of *N*-propargyl-1-aminoindan compounds as inhibitors of B-form of monoamine oxidase enzyme, EP 436492, CAN 115:158747, 1991.
86. Youshko, M.I., van Rantwijk, F., and Sheldon, R.A., Enantioselective acylation of chiral amines catalyzed by aminoacylase I, *Tetrahedron Asymmetry*, 12(23), 3267–3271, 2001.
87. van Langen, L.M., Oosthoek, N.H.P., Guranda, D.T., et al., Penicillin acylase-catalyzed resolution of amines in aqueous organic solvents, *Tetrahedron Asymmetry*, 11(22), 4593–4600, 2000.
88. Takayama, S., Lee, S.T., Hung, S.-C., et al., Designing enzymatic resolution of amines, *Chem. Commun.*, (2), 127–128, 1999.
89. Hacking, M.A.P.J., van Rantwijk, F., and Sheldon, R.A., Lipase catalyzed reactions of aliphatic and arylaliphatic carbonic acid esters, *J. Mol. Catal., B Enzym.*, 9(4–6), 201–208, 2000.
90. Choi, Y.K., Kim, M.J., Ahn, Y., et al., Lipase/palladium-catalyzed asymmetric transformations of ketoximes to optically active amines, *Org. Lett.*, 3(25), 4099–4101, 2001.
91. Messina, F., Botta, M., Corelli, F., et al., Chiral azole derivatives, 3. Synthesis of the enantiomers of the potent aromatase inhibitor 1-[2-benzofuranyl(4-chlorophenyl)methyl]-1*H*-imidazole, *Tetrahedron Lett.*, 40(40), 7289–7292, 1999.
92. Messina, F., Botta, M., Corelli, F., et al., Resolution of (\pm)-1-aryl-2-propynylamines via acyl transfer catalyzed by *Candida antarctica* lipase, *J. Org. Chem.*, 64(10), 3767–3769, 1999.
93. Taylor K.M. and Snyder S.H., Amphetamine: differentiation by *d* and *l* isomers of behavior involving brain norepinephrine or dopamine, *Science*, 168(938), 1487–1489, 1970.
94. Hajos, G.T. and Garattini, S., A note on the effect of (+)- and (–)-amphetamine on lipid metabolism, *J. Pharm. Pharmacol.*, 25(5), 418–419, 1973.

95. González-Sabín, J., Gotor, V., and Rebolledo, F., CAL-B-catalyzed resolution of some pharmacologically interesting β -substituted isopropylamines, *Tetrahedron Asymmetry*, 13(12), 1315–1320, 2002.
96. Goument, B., Duhamel, L., and Mauge, R., Asymmetric syntheses of (*S*)-fenfluramine using sharpless epoxidation methods, *Tetrahedron*, 50(1), 171–188, 1994.
97. Fogagnolo, M., Giovannini, P.P., Guerrini, A., et al., Homochiral (*R*)- and (*S*)-1-heteroaryl- and 1-aryl-2-propanols via microbial redox, *Tetrahedron Asymmetry*, 9(13), 2317–2327, 1998.
98. Turgeon, J., Uprichard, A.C., Belanger, P.M., et al., Resolution and electrophysiological effects of mexiletine enantiomers, *J. Pharm. Pharmacol.*, 43(9), 630–635, 1991.
99. Gold, E.H., Chang, W., Cohen, M., et al., Synthesis and comparison of some cardiovascular properties of the stereoisomers of labetalol, *J. Med. Chem.*, 25(11), 1363–1370, 1982.
100. Guranda, D.T., van Langen, L.M., van Rantwijk, F., et al., Highly efficient and enantioselective enzymatic acylation of amines in aqueous medium, *Tetrahedron Asymmetry*, 12(11), 1645–1650, 2001.
101. Breen, G.F., Forth, M.A., and Popkin, M.E., Process for preparing 1-methyl-1,2,3,4-tetrahydroisoquinoline or a salt thereof, WO 2002088088, CAN 137:352906, 2002.
102. Breen, G.F., Enzymatic resolution of a secondary amine using novel acylating reagents, *Tetrahedron Asymmetry*, 15(9), 1427–1430, 2004.
103. Njoroge, F.G., Taveras, A.G., Kelly, J., et al., (+)-4-[2-[4-(8-Chloro-3,10-dibromo-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11(*R*)-yl)-1-piperidinyl]-2-oxo-ethyl]-1-piperidinecarboxamide (SCH-66336): a very potent Farnesyl protein transferase inhibitor as a novel antitumor agent, *J. Med. Chem.*, 41(24), 4890–4902, 1998.
104. Morgan, B., Zaks, A., Dodds, D.R., et al., Enzymatic kinetic resolution of piperidine atropisomers: synthesis of a key intermediate of the farnesyl protein transferase inhibitor, SCH66336, *J. Org. Chem.*, 65(18), 5451–5459, 2000.
105. Lucet, D., Le Gall, T., and Mioskowski, C., The chemistry of vicinal diamines, *Angew. Chem. Int. Ed. Engl.*, 37(19), 2580–2627, 1998.
106. Michalson, E.T. and Szmuszkowicz, J., Medicinal agents incorporating the 1,2-diamine functionality, *Prog. Drug Res.*, 33, 135–149, 1989.
107. Alfonso, I., Astorga, C., Rebolledo, F., et al., Sequential biocatalytic resolution of (\pm)-*trans*-cyclohexane-1,2-diamine. Chemoenzymic synthesis of an optically active polyamine, *Chem. Commun.*, (21), 2471–2472, 1996.
108. De Costa, B.R., Bowen, W.D., Hellewell, S.B., et al., Alterations in the stereochemistry of the κ -selective opioid agonist U50,488 result in high-affinity σ ligands, *J. Med. Chem.*, 32(8), 1996–2002, 1989.
109. González-Sabín, J., Gotor, V., and Rebolledo, F., Chemoenzymatic preparation of optically active *trans*-cyclohexane-1,2-diamine derivatives: an efficient synthesis of the analgesic U-(–)-50,488, *Chem. Eur. J.*, 10(22), 5788–5794, 2004.
110. de Zoete, M.C., Kock-van Dalen, A.C., van Rantwijk, F., et al., Ester ammonolysis: a new enzymic reaction, *J. Chem. Soc. Chem. Commun.*, (24), 1831–1832, 1993.
111. Ghosh, A., Sieser, J.E., Caron, S., et al., Synthesis of the kappa-agonist CJ-15,161 via a palladium-catalyzed cross-coupling reaction, *Chem. Commun.*, (15), 1644–1645, 2002.
112. Gottschlich, R., Ackermann, K.A., Barber, A., et al., EMD 61753 as a favorable representative of structurally novel arylacetamido-type κ opiate receptor agonists, *Bioorg. Med. Chem. Lett.*, 4(5), 677–682, 1994.
113. Naylor, A., Judd, D.B., Scopes, D.I.C., et al., 4-[(Alkylamino)methyl]furo[3,2-*c*]pyridines: a new series of selective κ -receptor agonists, *J. Med. Chem.*, 37(14), 2138–2144, 1994.
114. Semple, G., Andersson, B.-M., Chhajlani, V., et al., Synthesis and biological activity of kappa opioid receptor agonists. Part 2: preparation of 3-aryl-2-pyridone analogues generated by solution- and solid-phase parallel synthesis methods, *Bioorg. Med. Chem. Lett.*, 13(6), 1141–1145, 2003.
115. García-Urdiales, E., Rebolledo, F., and Gotor, V., Enzymatic ammonolysis of ethyl (\pm)-4-chloro-3-hydroxybutanoate. Chemoenzymatic syntheses of both enantiomers of pyrrolidin-3-ol and 5-(chloromethyl)-1,3-oxazolidin-2-one, *Tetrahedron Asymmetry*, 10(4), 721–726, 1999.

116. Puertas, S., Rebolledo, F., and Gotor, V., Enantioselective enzymic aminolysis and ammonolysis of dimethyl 3-hydroxyglutarate. Synthesis of (*R*)-4-amino-3-hydroxybutanoic acid, *J. Org. Chem.*, 61(17), 6024–6027, 1996.
117. Zhang, L.-h., Chung, J.C., Costello, T.D., et al., The enantiospecific synthesis of an isoxazoline. A RGD mimic platelet GPIIb/IIIa antagonist, *J. Org. Chem.*, 62(8), 2466–2470, 1997.
118. Sigmund, A.E., McNulty, K.C., Nguyen, D., et al., Enantioselective enzymatic aminolysis of a racemic 2-isoxazolylacetate alkyl ester, *Can. J. Chem.*, 80(6), 608–612, 2002.
119. Zmijewski, M.J., Briggs, B.S., Thompson, A.R., et al., Enantioselective acylation of a beta-lactam intermediate in the synthesis of loracarbef using penicillin G amidase, *Tetrahedron Lett.*, 32(13), 1621–1622, 1991.
120. Basso, A., Braiuca, P., De Martin, L., et al., D-Phenylglycine and D-4-hydroxyphenylglycine methyl esters via penicillin G acylase catalysed resolution in organic solvents, *Tetrahedron Asymmetry*, 11(8), 1789–1796, 2000.
121. Petersen, M. and Sauter, M., Biotechnology in the fine-chemicals industry. Cyclic amino acids by enantioselective biocatalysis, *Chimia*, 53(12), 608–612, 1999.
122. Martin, J., Deagostino, A., Perrio, C., et al., Syntheses of *R* and *S* isomers of AF-DX 384, a selective antagonist of muscarinic M₂ receptors, *Bioorg. Med. Chem.*, 8(3), 591–600, 2000.
123. Liljeblad, A., Lindborg, J., Kanerva, A., et al., Enantioselective lipase-catalyzed reactions of methyl pipicolinate: transesterification and *N*-acylation, *Tetrahedron Lett.*, 43(13), 2471–2474, 2002.
124. Pohlmann, J., Lampe, T., Shimada, M., et al., Pyrrolidinedione derivatives as antibacterial agents with a novel mode of action, *Bioorg. Med. Chem. Lett.*, 15(4), 1189–1192, 2005.
125. Davies, S.G. and Dixon, D.J., Asymmetric syntheses of moiramide B and andrimid, *J. Chem. Soc. Perkin Trans.*, 1(17), 2635–2644, 1998.
126. Gedey, S., Liljeblad, A., Lazar, L., et al., Preparation of highly enantiopure β-amino esters by *Candida antarctica* lipase A, *Tetrahedron Asymmetry*, 12(1), 105–110, 2001.
127. Cossy, J., Schmitt, A., Cinquin, C., et al., A very short, efficient and inexpensive synthesis of the prodrug form of SC-54701A a platelet aggregation inhibitor, *Bioorg. Med. Chem. Lett.*, 7(13), 1699–1700, 1997.
128. Yamanaka, T., Ohkubo, M., Takahashi, F., et al., An efficient synthesis of the orally-active GpIIb/IIIa antagonist FR184764, *Tetrahedron Lett.*, 45(13), 2843–2845, 2004.
129. Landis, B.H., Mullins, P.B., Mullins, K.E., et al., Kinetic resolution of β-amino esters by acylation using immobilized penicillin amidohydrolase, *Org. Proc. Res. Dev.*, 6(4), 539–546, 2002.
130. Kanerva, L.T., Csomós, P., Sundholm, O., et al., Approach to highly enantiopure β-amino acid esters by using lipase catalysis in organic media, *Tetrahedron Asymmetry*, 7(6), 1705–1716, 1996.

8 Dynamic Kinetic Resolution and Asymmetric Transformations by Enzyme–Metal Combinations

Mahn-Joo Kim, Yangsoo Ahn, and Jaiwook Park

CONTENTS

8.1	Introduction	249
8.2	Dynamic Kinetic Resolution by Enzyme–Metal Combinations	250
8.2.1	Dynamic Kinetic Resolution by Lipase–Ruthenium Combination	250
8.2.1.1	Dynamic Kinetic Resolution of Secondary Alcohols	250
8.2.1.2	Dynamic Kinetic Resolution of Functionalized Alcohols	252
8.2.2	Dynamic Kinetic Resolution by Subtilisin–Ruthenium Combination	254
8.2.2.1	Dynamic Kinetic Resolution of Secondary Alcohols	254
8.2.3	Dynamic Kinetic Resolution by Lipase–Palladium Combination	257
8.2.3.1	Dynamic Kinetic Resolution of Allyl Acetates	257
8.2.3.2	Dynamic Kinetic Resolution of Amines	258
8.3	Asymmetric Transformations by Enzyme–Metal Combo-Catalysis	260
8.3.1	Asymmetric Transformations by Lipase–Ruthenium Combination	260
8.3.1.1	Asymmetric Reductive Acetylation of Ketones	260
8.3.1.2	Asymmetric Hydrogenation of Enol Acetates	264
8.3.2	Asymmetric Transformations by Lipase–Palladium Combination	264
8.3.2.1	Asymmetric Reductive Acetylation of Ketoximes	264
8.4	Conclusion	269
	References	269

8.1 INTRODUCTION

The methods for the preparation of optically active compounds are of great importance in pharmaceutical and fine chemical industries [1]. One popular method is the resolution of racemic mixtures by enzymes such as lipases and esterases [2–16]. This method has been widely used for the preparation of optically active alcohols, acids, and their esters. However, the enzymatic resolution method has an intrinsic limitation; the yield cannot exceed 50% for a single enantiomer. Thus, the resolution is usually accompanied by additional processes such as separation, racemization, and recycling of unwanted enantiomers. The limitation, however, can be overcome if kinetic resolution could be transformed to dynamic kinetic resolution

(DKR) by coupling with a racemization reaction for the *in situ* conversion of unwanted enantiomers to products [17].

Recently, several groups have reported the use of a metal complex as the racemizing catalyst along with an enzyme in the DKR [18–26]. Allen and Williams employed palladium as the racemizing catalyst together with lipase in the DKR of allyl acetates [27] and rhodium in the DKR of secondary alcohols [28]. Reetz and Schimossek used palladium for the racemization of 1-phenylethylamine in its DKR with lipase [29]. Later, the Bäckvall group reported the DKRs of secondary alcohols employing a ruthenium complex for the racemization. Recently, we developed a new Ru-based catalyst for the efficient racemization of secondary alcohols at room temperature [30]. The room temperature DKRs of secondary alcohols with the Ru catalyst were successfully performed with both lipase and subtilisin to provide (*R*)- and (*S*)-products, respectively, in good yields. The enzyme–metal combination strategy has also been applied to the asymmetric transformations of prochiral ketones and ketoximes.

This chapter covers the recent developments in the DKR and the asymmetric transformations by enzyme–metal combinations.

8.2 DYNAMIC KINETIC RESOLUTION BY ENZYME–METAL COMBINATIONS

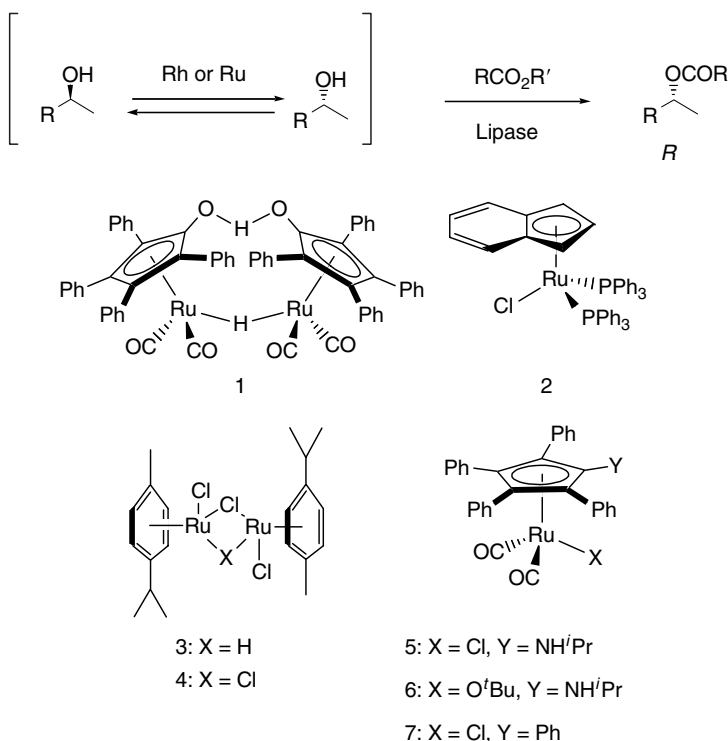
8.2.1 DYNAMIC KINETIC RESOLUTION BY LIPASE–RUTHENIUM COMBINATION

8.2.1.1 Dynamic Kinetic Resolution of Secondary Alcohols

The first DKR of secondary alcohols, reported by the Williams group in 1996 [27], was performed by the combination of lipase as the resolving catalyst and rhodium complex as the racemizing catalyst (Scheme 8.1). The lipase-Rh catalyzed DKR of 1-phenylethanol using vinyl acetate as an acyl donor gave a modest result of 76% conversion and 80% ee. Later, Bäckvall et al. reported a significantly improved procedure using a diruthenium complex **1**, and an immobilized and thermally stable lipase (*Candida antarctica* lipase B, CALB; Novozym 435). The reactions performed at elevated temperature (70°C) gave excellent results in the presence of a special acyl donor *p*-chlorophenyl acetate (PCPA). Popular acyl donors such as vinyl acetate and isopropenyl acetate were incompatible with the racemizing catalyst [31–33]. The DKR of 1-phenylethanol by the procedure gave optically pure (*R*)- α -phenylethyl acetate (>99.5% ee) in a high yield (100% conversion, 92% isolated yield) (Table 8.1). However, the DKR required a stoichiometric amount of acetophenone that acts as a hydrogen acceptor for racemization, otherwise a large amount (>20%) of acetophenone was formed by decreasing the yield of (*R*)- α -phenylethyl acetate [32].

We found that an indenylruthenium complex **2** racemized secondary alcohols without the aid of ketones during DKR [34]. The DKR with **2**, however, required a catalytic amount of triethylamine and molecular oxygen to activate **2**. The combination of an immobilized *Pseudomonas cepacia* lipase (PCL, Lipase PS-C of Amano Enzyme Co., Japan) and **2** at 60°C was effective for the DKR of benzylic alcohols but was less effective for aliphatic alcohols (Table 8.2).

We then found a commercially available cymene–ruthenium complex **3** and its activated hydride form **4** as good racemizing catalysts. The activated complex **4**, in particular, showed satisfactory performances in the DKR of aliphatic alcohols as well as benzylic alcohols (Table 8.3). A noticeable feature of the catalyst is the high racemizing activity toward allylic alcohols [35], so that their DKR can be performed at room temperature to provide high yields of products with excellent optical purities (Table 8.4). Additional feature is its good activity in ionic liquids such as [EMIm]BF₄ and [BMIm]PF₆ ([EMIm] = 1-ethyl-3-methylimidazolium, [BMIm] = 1-butyl-3-methylimidazolium) [36–43]. The DKRs in the ionic liquids were also



SCHEME 8.1 DKR of secondary alcohols.

possible at room temperature wherein the racemizing catalyst and enzyme were reusable after the products were extracted with ether (Table 8.5) [44].

In an effort to develop racemizing catalysts that are active at room temperature, we synthesized a novel aminocyclopentadienyl ruthenium chloride complex **5** that transforms to its active form **6** upon *in situ* treatment with potassium *t*-butoxide [30]. The *in situ* generated species **6** displayed high activity in the DKR of aliphatic alcohols, as well as aromatic alcohols at room temperature (Table 8.6). Interestingly, it can be used with isopropenyl acetate that is incompatible with other ruthenium catalysts **1–4**. Used in the previous DKRs, isopropenyl acetate is more practical than PCPA; it is readily available, more active than PCPA, and is easily separable from the DKR products [45]. Although the

TABLE 8.1
DKR of 1-Phenylethanol with 1

Acyl Donor	ee (%)	Yield ^a (%)
Vinyl acetate	>99	50
Isopropenyl acetate	>99	72
4-Chlorophenyl acetate	>99	100

^a Determined by ¹H NMR and GC.

Note: The reactions were performed on a 2-mmol scale in 5 mL of *t*-BuOH with 2 mol% of complex **1**, 1 equivalent of acetophenone, 50 mg of Novozym 435, and acyl donor at 70°C under Ar atmosphere.

TABLE 8.2
DKR of Secondary Alcohols with 2

Substrate	Product	ee ^a (%)	Yield ^b (%)
		96	86
		99	82
		99	98
		82	88
		97	60

^a Measured by HPLC equipped with a chiral column.

^b Determined by ¹H NMR.

Note: The reactions were performed on a 0.25-mmol scale in 2-mL of CH₂Cl₂ with 5 mol% of complex **2**, 5 mol% of O₂, 3 equivalent of triethylamine, 3 equivalent of PCPA, and 40 mg of PCL at 60°C for 43 h.

mechanism for the catalytic racemization is not clear yet, according to our mechanistic studies, the amino group in **5** or **6** seems to be crucial for the racemization, while recent report by the Bäckvall group suggests a different pathway [46–48]. The Bäckvall group used a modified complex **7** as the efficient racemizing catalyst, which is similar to **5** but without an amino group.

A new catalyst system of [TosN(CH₂)₂NH₂] RuCl(*p*-cymene) and 2,2,6,6-tetramethyl-1-piperidinyloxy was reported by Sheldon et al. for the DKR of alcohols [49]. However, it was tested only for 1-phenylethanol to afford 1-phenylethyl acetate in 76% yield.

8.2.1.2 Dynamic Kinetic Resolution of Functionalized Alcohols

The DKR of functionalized alcohols such as diols, hydroxy esters, hydroxy aldehydes, azido alcohols, and hydroxy nitriles were well performed by lipase–ruthenium bicatalysis. The DKR of diols was achieved with diruthenium catalyst **1** and CALB in the presence of PCPA to give the corresponding diacetates of (*R,R*)-configuration from the mixture of *dl*- and *meso*-isomers (Table 8.7) [50,51]. The DKR of rigid benzylic diols with **1** gave better results in terms of *de* compared to those of more flexible aliphatic diols, reflecting that lipase displays higher stereoselectivity toward benzylic diols than aliphatic diols.

TABLE 8.3
DKR of Secondary Alcohols with 4

Substrate	Product	ee (%)	Yield (%)
		94	95
		99	93
		99	93
		>99	85

Note: The reactions were performed in CH_2Cl_2 with 4 mol% of complex **4**, 1 equivalent of triethylamine, 1.5 equivalent of PCPA, and PCL at 40°C under Ar atmosphere.

The DKR of hydroxy esters were also accomplished with PCL and **1** at 60°C ~70°C [52–55]. In most cases the enantioselectivities were good, but the yields were moderate (Table 8.8 and Table 8.9). The use of H_2 was necessary in the DKR of γ - and δ -hydroxy esters to suppress the formation of ketones.

The DKR of small functionalized alcohols such as 2-hydroxybutanoic acid, 2-hydroxypropanal, and 1,2-propanediol was carried out after the protection of the terminal groups with a bulky group, because the bulky protecting groups enhanced the enantioselectivity of enzyme [56]. In the DKR of hydroxy acids, *t*-butyl group was the best protecting group for the carboxylic acid functionality (Table 8.10). The trityl group was a proper choice for the protection of primary alcohols in diols such as 1,2-propanediol, 1,2-butanediol, and 1,3-butanediol [56]. For example, 1,2-benzenedimethanol was used for protecting the formyl groups of α - and β -hydroxy aldehydes (Table 8.11) [56]. High enantiomeric excesses (95% and more) were obtained in the DKRs of the protected diols and hydroxy aldehydes. Similarly, 2,6-dimethyl-4-heptanol was used as a hydrogen source to suppress the formation of the oxidized side products.

The DKR of β -azidoalcohols [55] and β -hydroxynitriles [57–59] were also accomplished by employing **1** and CALB, with PCPA as the acyl donor. The DKR of β -azidoalcohols were performed at 60°C while that of β -hydroxynitriles required a higher temperature (100°C) to increase the racemization rate. The optical purities of products were satisfactory in all cases. In the case of β -hydroxynitriles, dehydrogenation lowered the yield.

TABLE 8.4
DKR of Allyl Alcohols with 4

Substrate	Product	ee ^a (%)	Yield ^b (%)
		>99	84
		99	91
		99	85
		99	92
		95	90
		>99	85

^a Measured by HPLC or GC equipped with a chiral column.

^b Determined by ¹H NMR.

Note: The reactions were performed on a 0.5-mmol scale in 2 mL of CH₂Cl₂ with 4 mol% of complex **4**, 1 equivalent of triethylamine, 1.5 equivalent of PCPA, and 75 mg of PCL at room temperature for 2 d.

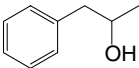
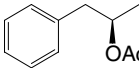
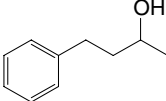
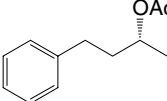
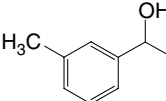
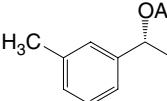
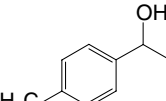
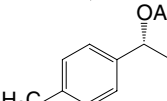
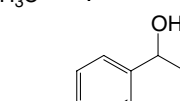
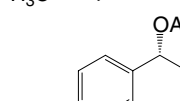
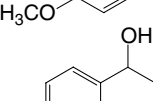
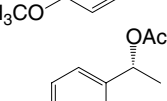
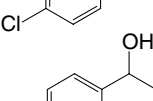
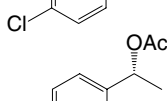
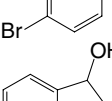
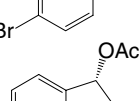
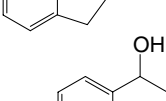
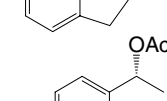
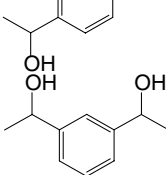
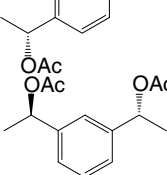
8.2.2 DYNAMIC KINETIC RESOLUTION BY SUBTILISIN–RUTHENIUM COMBINATION

8.2.2.1 Dynamic Kinetic Resolution of Secondary Alcohols

The lipase-catalyzed DKRs provide only (*R*)-products. To obtain (*S*)-products, we need an enzyme with a complementary (*S*)-stereoselectivity. We surveyed (*S*)-selective enzymes compatible with the racemizing catalyst **6**. Though subtilisin was suitable, its commercial form was not applicable to DKR due to its low enzymatic activity and instability in nonaqueous medium. However, we succeeded in enhancing its activity and stability by treating it with a surfactant before use. Thus, the DKR with surfactant-treated subtilisin, **6**, and trifluoroethyl butanoate as an acylating agent gave the (*S*)-products in good yields with high optical purities at room temperature (Scheme 8.2 and Table 8.12) [60].

The (*S*)-selective DKR of alcohols with subtilisin was also possible in ionic liquid at room temperature (Table 8.13) [44]. In this case, the cymene–ruthenium complex **4** was used as the racemization catalyst. In general, the optical purities of (*S*)-esters were lower than those of (*R*)-esters as described in Table 8.5.

TABLE 8.5
DKR of Secondary Alcohols with 4 in [BMIm]PF₆

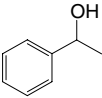
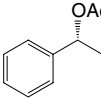
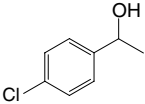
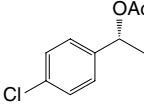
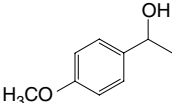
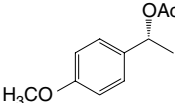
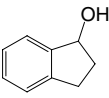
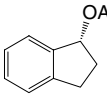
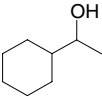
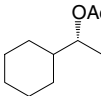
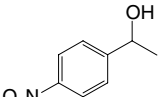
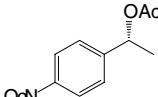
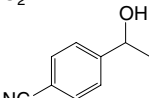
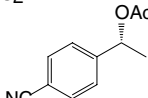
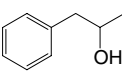
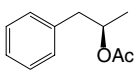
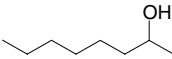
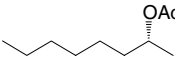
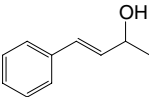
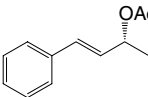
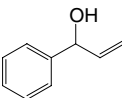
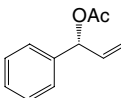
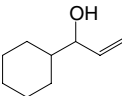
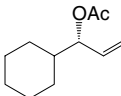
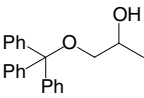
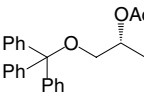
Substrate	Product	ee ^a (%)	Yield ^b (%)
		99	85
		99	85
		98	87
		99	87
		99	85
		99	87
		99	92
		99	85
		99 (de 99%)	87
		99 (de 97%)	86

^a Measured by HPLC equipped with a chiral column.

^b Determined by ¹H NMR.

Note: The reactions were performed on a 0.3-mmol scale in 1 mL of [BMIm]PF₆ with 8 mol% of complex **4**, 1 equivalent of triethylamine, 3 equivalent of 1,1,1-trifluoroethyl acetate, and 45 mg of LPS-TN-M at room temperature for 2–4 d under Ar atmosphere.

DKR of Secondary Alcohols with **6**

Substrate	Product	ee ^a (%)	Yield ^b (%)
		>99	95
		>99	94
		>99	90
		95	89
		>99	86 ^c
		>99	97
		>99	95
		>99	90
		91	89 ^c
		98	93
		81	62
		>99	90
		99	97

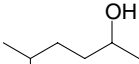
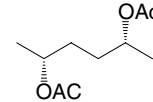
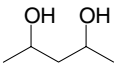
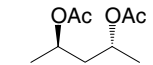
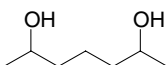
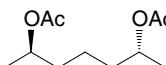
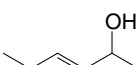
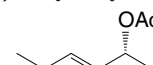
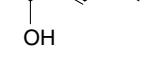
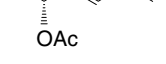
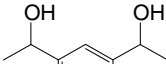
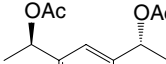
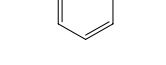
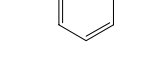
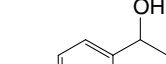
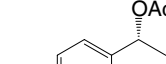
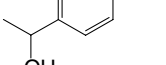
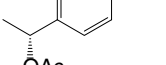
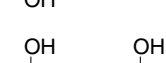
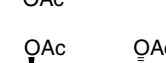
^a Measured by HPLC equipped with a chiral column.

^b Isolated yields.

^c Measured by GC equipped with a chiral column.

Note: The reactions were performed on a 1.0-mmol scale in 3.2 mL of toluene with isopropenyl acetate (1.5 equivalent), Novozym 435 (3 mg), Na₂CO₃ (1.0 equivalent), **5** (4 mol%), and potassium *tert*-butoxide (5 mol%) at 25°C for 2–6 d under Ar atmosphere.

TABLE 8.7
DKR of Diols

Substrate	Catalyst	Product	ee ^a (%)	(<i>R,R</i>)/meso ^a	Yield ^b (%)
	1		>99	86/14	63
	1		>99	38/62	90
	1		>97	90/10	63
	1		>99	74/26	43
	1		>99	98/2	76
	6		>99	99/1	95
	1		>99	98/2	77
	6		>99	98/2	94
	1		>99	100/0	78
	1		>96	89/11	64

^a Measured by HPLC or GC equipped with a chiral column.

^b Isolated yields.

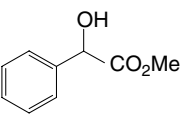
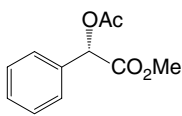
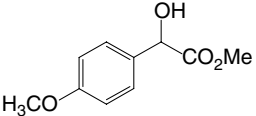
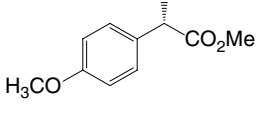
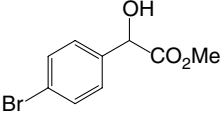
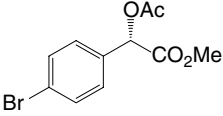
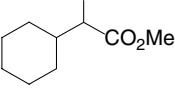
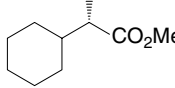
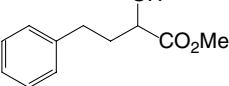
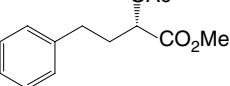
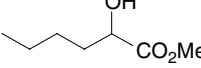
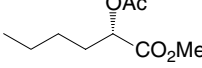
Note: The reactions were performed on a 0.5-mmol scale in 1 mL of toluene with PCPA (3 equivalent), Novozym 435 (30 mg), and **1** (4 mol %) at 70°C for 1–2 d under Ar atmosphere.

8.2.3 DYNAMIC KINETIC RESOLUTION BY LIPASE–PALLADIUM COMBINATION

8.2.3.1 Dynamic Kinetic Resolution of Allyl Acetates

The first DKR of allyl acetates was accomplished through coupled Pd-catalyzed racemization and enzymatic hydrolysis of allyl acetates in a buffer solution [27]. However, the DKR was limited to cyclohexenyl acetates to give symmetrical palladium–allyl intermediates. Among them, only 2-phenyl-2-cyclohexenyl acetate was resolved with good results (96% conversion, 81% yield, and 96% ee) (Scheme 8.3).

TABLE 8.8
DKR of α -Hydroxy Esters with **1**

Substrate	Product	ee ^a (%)	Yield ^b (%)
		94	80
		94	76
		98	69
		98	80
		30	62
		80	60

^a Measured by HPLC or GC equipped with a chiral column.

^b Isolated yields.

Note: The reactions were performed on a 0.25-mmol scale in 1.25 mL of cyclohexane with PCPA (2 equivalent), LPS-C (15 mg), and **1** (2 mol%) at 60°C for 2–3 d under

We improved the DKR of allyl acetates by replacing enzymatic hydrolytic reaction with enzymatic transesterification reaction and by employing Pd(PPh₃)₄ and 1,1'-bis(diphenylphosphino)ferrocene (dppf) as the racemizing catalyst system (Scheme 8.4). The DKR reactions were performed with 2-propanol as an acyl acceptor in tetrahydrofuran (THF) [61]. The use of the chelating ligand (dppf) decreased the formation of byproducts (1,3-dienes). Various acyclic allylic acetates were transformed to their corresponding allylic alcohols at room temperature with good yields and excellent enantioselectivities (Table 8.14).

8.2.3.2 Dynamic Kinetic Resolution of Amines

Reetz et al. reported for the first time the DKR of 1-phenylethylamine by employing palladium on carbon and CALB [29]. However, the DKR required a very long reaction time (8 d) at 50°C ~55°C and provided a poor isolated yield (60%) (Scheme 8.5). Recently, Bäckvall et al. reported that diruthenium complex **1** racemizes aromatic amines at 110°C in toluene, but the racemization conditions were not applicable to the DKR involving enzymes [62].

TABLE 8.9
DKR of β -Hydroxy Esters with 1

Substrate	Product	ee ^a (%)	Yield ^b (%)
		95	76
		99	74
		96	80
		70	82

^a Measured by GC equipped with a chiral column.

^b Isolated yields.

Note: The reactions were performed on a 0.8-mmol scale in 8 mL of TBME with PCPA (3 equivalent), LPS-C (48 mg), and **1** (6 mol%) at 60°C for 6 d under Ar atmosphere.

TABLE 8.10
DKR of 2-Hydroxybutanoic Acid Esters with 1

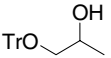
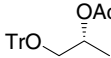
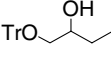
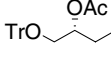
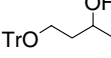
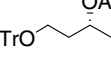
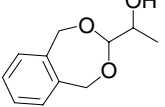
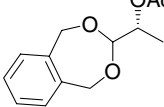
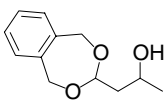
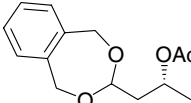
Substrate	Product	ee ^a (%)	Yield ^b (%)
		86	88
		93	91
		94	92
		>99	88

^a Measured by HPLC equipped with a chiral column.

^b Isolated yields.

Note: The reactions were performed on a 0.3-mmol scale in 1 mL of toluene with PCPA (6.5 equivalent), PCL (24 mg), and **1** (10 mol%) at 70°C for 4 d under Ar atmosphere.

TABLE 8.11
DKR of Protected Diols and Hydroxy Aldehydes

Substrate	Product	ee ^a (%)	Yield ^b (%)
		>99	96
		99	91
		95	97
		98	95
		96	90

^a Measured by HPLC equipped with a chiral column.

^b Isolated yields.

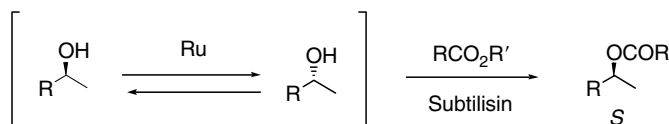
Note: The reactions were performed on a 0.3-mmol scale in 1 mL of toluene with PCPA (6.5 equivalent), PCL (24 mg), and **1** (10 mol%) at 70°C for 4 d under Ar atmosphere.

8.3 ASYMMETRIC TRANSFORMATIONS BY ENZYME–METAL COMBO-CATALYSIS

8.3.1 ASYMMETRIC TRANSFORMATION BY LIPASE–RUTHENIUM COMBINATION

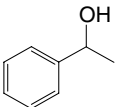
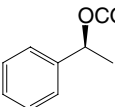
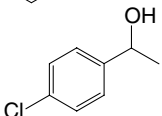
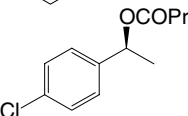
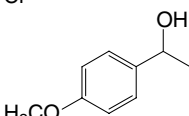
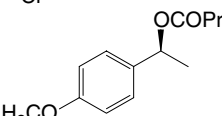
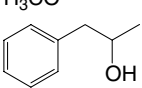
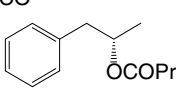
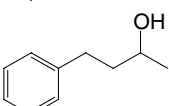
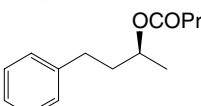
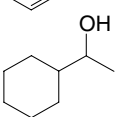
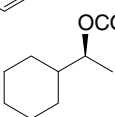
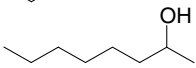
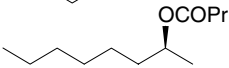
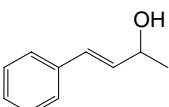
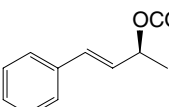
8.3.1.1 Asymmetric Reductive Acetylation of Ketones

The catalytic alcohol racemization with diruthenium catalyst **1** is based on a reversible transfer hydrogenation mechanism including ketone as an oxidized intermediate. Therefore, the formation of ketone as a major side product is inevitable in the DKR of secondary alcohols with **1**. To circumvent the problem of ketone formation, we envisioned a novel DKR process starting from ketone ([Scheme 8.6](#)). A key factor of this process was the selection of hydrogen donors compatible with the DKR conditions. Finally, 2,6-dimethylheptan-4-ol, which cannot be acylated by lipases, and molecular hydrogen was chosen as hydrogen donors [63,64]. Asymmetric reductive acetylation of ketones under 1 atm of hydrogen was performed in ethyl acetate as an acyl donor and solvent, which needed a long



SCHEME 8.2 (*S*)-Selective DKR of secondary alcohols.

TABLE 8.12
(S)-Selective DKR of Secondary Alcohols by Subtilisin–Ru
Combination

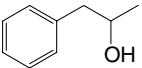
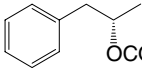
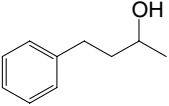
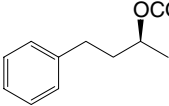
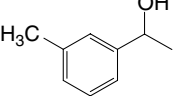
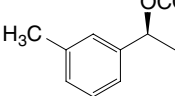
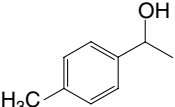
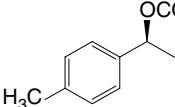
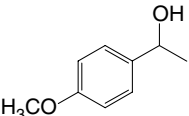
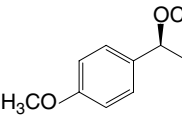
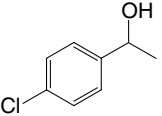
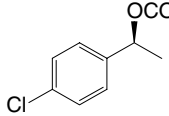
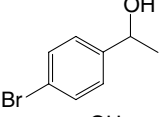
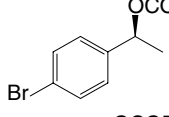
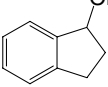
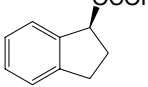
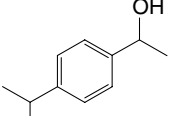
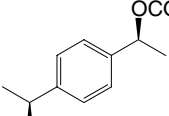
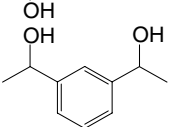
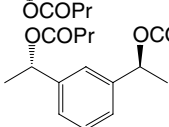
Substrate	Product	ee ^a (%)	Yield ^b (%)
		92	95
		99	92 (90)
		94	93 (91)
		92	77 (76)
		98	80 (78)
		98	80 (74)
		98	77 (67)
		95	90 (90)

^a Measured by HPLC or GC equipped with a chiral column.

^b Determined by ¹H NMR (isolated yields in parentheses).

Note: The reactions were performed on a 0.3-mmol scale in 1 mL of THF with 2,2,2-trifluoroethyl butyrate (1.7 equivalent), the subtilisin (7.5 mg), Na₂CO₃ (63.6 mg), **5** (4 mol%), and potassium *tert*-butoxide (5 mol%) at 25°C for 3 d

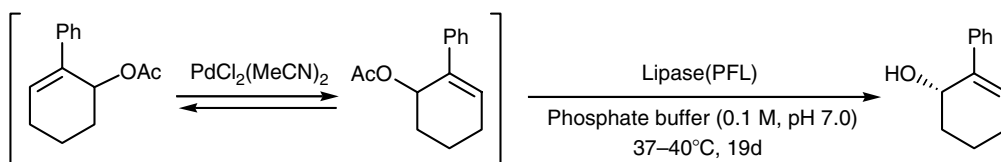
TABLE 8.13
(S)-Selective DKR of Secondary Alcohols in [BMIm]PF₆

Substrate	Product	ee ^a (%)	Yield ^b (%)
		97	89
		97	90
		85	90
		85	90
		99	80
		87	92
		82	91
		86	84
		86 (de 52%)	78
		96 (de 63%)	83

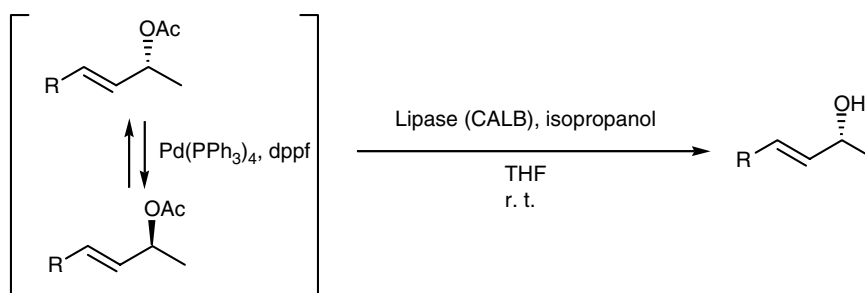
^a Measured by HPLC equipped with a chiral column.

^b Determined by ¹H NMR.

Note: The reactions were performed on a 0.3-mmol scale in 1 mL of [BMIm]PF₆ with 8 mol% of complex **4**, 1 equivalent of triethylamine, 3 equivalent of 1,1,1-trifluoroethyl butyrate, and 20 mg of subtilisin-CLEC at room temperature for 6 d under Ar atmosphere.



SCHEME 8.3 DKR of 2-phenyl-2-cyclohexenyl acetate.



SCHEME 8.4 DKR of allyl acetates.

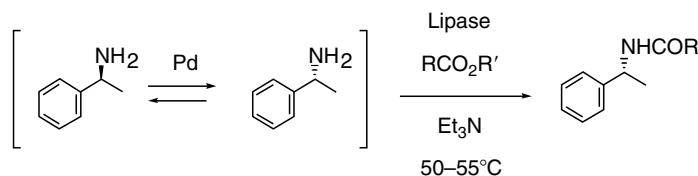
TABLE 8.14
DKR of Allyl Acetates by Lipase–Pd Combination

Substrate	Product	ee ^a (%)	Yield ^b (%)
		98	83
		97	77
		98	82
		>99	87
		98	70

^a Measured by HPLC equipped with a chiral column.

^b Determined on the basis of HPLC results.

Note: The reactions were performed on a 0.53-mmol scale in 2 mL of THF with 5 mol% of Pd(PPh₃)₄, 15 mol% equivalent of dppf, 0.4 mL of i-PrOH, and 200 mg of CAL at room temperature for 2 d under Ar atmosphere.



SCHEME 8.5 DKR of 1-phenylethylamine.

reaction time (96 h). Ethanol formation from ethyl acetate did not cause critical problem, and various ketones were transformed successfully into the corresponding chiral acetates (Table 8.15) [64].

Asymmetric reductive acetylation process was also applicable to acetoxyaryl ketones [65]. For example, 3'-acetoxyacetophenone was transformed to (*R*)-1-(3-hydroxyphenyl)ethyl acetate under 1 atm of H₂ in 95% yield. The overall reaction seems to be a simple asymmetric reductive intramolecular acyl migration. In fact, however, it is the result from nine catalytic steps: two ruthenium-catalyzed reductions, two ruthenium-catalyzed epimerizations, three lipase-catalyzed deacylations, and two lipase-catalyzed acylations (Scheme 8.7). This process was applicable to a wide range of acyloxyphenyl ketones (Table 8.16). In most cases, high yields and excellent optical purities were obtained.

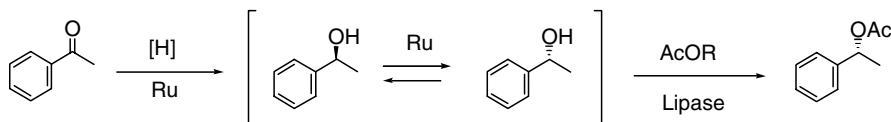
8.3.1.2 Asymmetric Hydrogenation of Enol Acetates

After succeeding in the asymmetric reductive acylation of ketones, we studied to see if enol acetates can be used as acyl donors and precursors of ketones at the same time through deacylation and keto-enol tautomerization (Scheme 8.8). The overall reaction thus corresponds to the asymmetric reduction of enol acetate. For example, 1-phenylvinyl acetate was transformed to (*R*)-1-phenylethyl acetate by CALB and diruthenium complex **1** in the presence of 2,6-dimethyl-4-heptanol in 89% yield (98% ee) [63]. Molecular hydrogen (1 atm) was almost equally effective for the transformation (86% yield, 96% ee) [64]. A broad range of enol acetates were prepared from ketones and were successfully transformed to the corresponding (*R*)-acetates under 1 atm of H₂ (Table 8.17). From unsymmetrical aliphatic ketones, enol acetates were obtained as the mixtures of regio- and geometrical isomers. Notably, however, the efficiency of the process was little affected by the isomeric composition of the enol acetates.

8.3.2 ASYMMETRIC TRANSFORMATIONS BY LIPASE-PALLADIUM COMBINATION

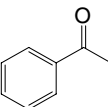
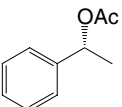
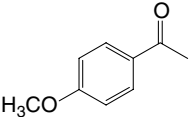
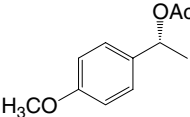
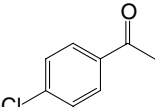
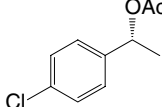
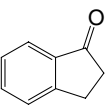
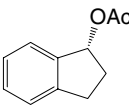
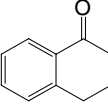
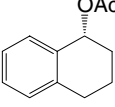
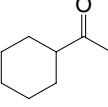
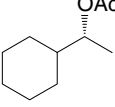
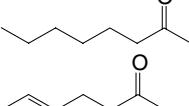
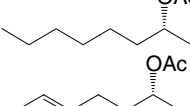
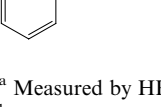
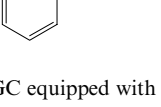
8.3.2.1 Asymmetric Reductive Acetylation of Ketoximes

The strategy for the asymmetric reductive acylation of ketones was extended to ketoximes by coupling the reduction of ketoximes to the DKR of amines (Scheme 8.9). The asymmetric



SCHEME 8.6 Asymmetric reductive acetylation of ketones.

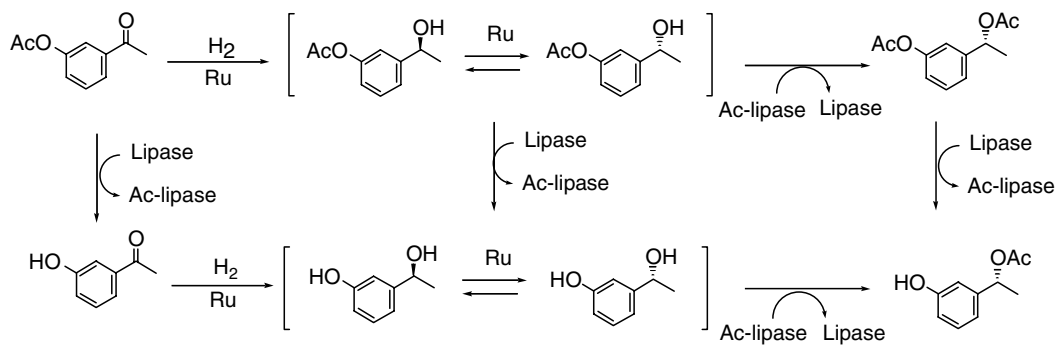
TABLE 8.15
Asymmetric Reductive Acetylation of Ketones

Substrate	Product	ee ^a (%)	Yield ^b (%)
		96	81
		99	85
		97	72
		99	89
		99	87
		90	87
		91	87
		72	83

^a Measured by HPLC or GC equipped with a chiral column.

^b Isolated yields.

Note: The reactions were performed on a 1.0-mmol scale in 3 mL of ethyl acetate with Novozym 435 (84 mg) and **1** (2 mol%) at 70°C for 2–4 d under 1 atm of H₂.



SCHEME 8.7 Reaction pathway for the asymmetric transformation of 3'-acetoxyacetophenone.

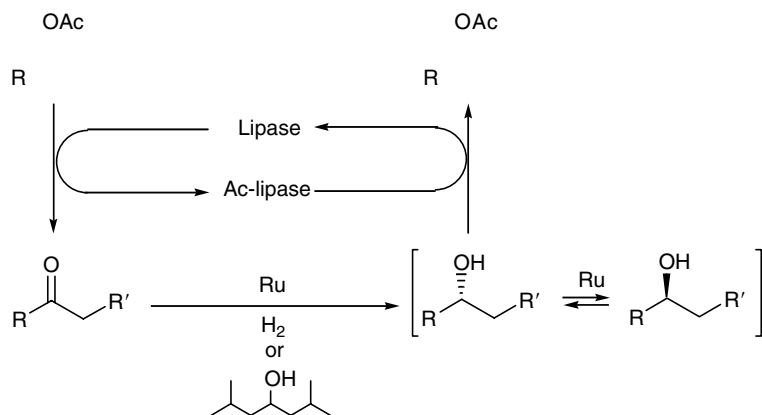
TABLE 8.16
Asymmetric Transformations of Acyloxyphenyl Ketones

Substrate	Product	ee ^a (%)	Yield ^b (%)
		98	95
		98	96
		93	94
		96	93
		89	88
		98	88
		96	92
		98	89

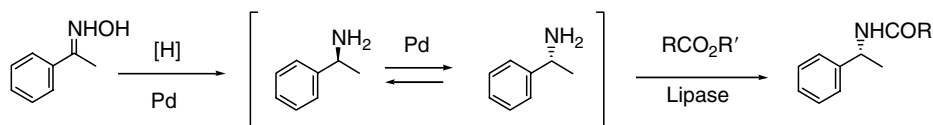
^a Measured by HPLC or GC equipped with a chiral column.

^b Isolated yields.

Note: The reaction were performed on a 0.56-mmol scale in 1.5 mL of toluene with LPS-D (25 mg), and **1** (0.022 mmol) at 70°C for 3d under Ar atmosphere.



SCHEME 8.8 Asymmetric hydrogenation of enol acetates.



SCHEME 8.9 Asymmetric reductive acetylation of ketoximes.

TABLE 8.17
Asymmetric Hydrogenation of Enol Acetates

Substrate	Product	ee ^a (%)	Yield ^b (%)
		98	89
		98	80
		97	91
		99	87
		79	90
		94	92
		99	94
		91	95

^a Measured by HPLC or GC equipped with a chiral column.

^b Isolated yields.

Note: The reaction were performed on a 1.0-mmol scale in 3 mL of toluene with Novozym 435(30 mg) and **1** (2 mol%) at 70°C for 2–4 d under 1 atm of H₂.

reactions of ketoximes were performed with CALB and Pd/C in the presence of hydrogen, diisopropylethylamine, and ethyl acetate in toluene at 60°C for 5 d (Table 8.18) [66]. In comparison to the direct DKR of amines, the yields of chiral amides increased significantly. The use of diisopropylethylamine to keep the reaction condition basic was a factor for the increase. However, the major factor would be the slow generation of amines, which maintains

TABLE 8.18
Asymmetric Reductive Acetylation of Ketoximes

Substrate	Product	ee ^a (%)	Yield ^b (%)
		98	80
		97	84
		94	81
		96	82
		98	76
		95	84
		97	70
		99	89

^a Measured by HPLC equipped with a chiral column.

^b Isolated yields.

Note: The reactions were performed on a 0.37-mmol scale in 3.7 mL of toluene with Novozym 435 (200 wt%), diisopropylethylamine (3 equivalent), ethyl acetate (2 equivalent), and Pd (66 wt%, 5% Pd/C) at 60°C for 5 d under 1 atm of H₂.

the amine concentration low enough to suppress side reactions including the reductive deamination. Disappointingly, this process is limited to benzylic amines. Low turnover frequencies also need to be overcome.

8.4 CONCLUSION

This chapter describes that enzyme–metal combo-catalysis provides a novel approach for the conversion of racemic substrates to single enantiomeric products. The key feature of this methodology is DKR by the combination of metal-catalyzed racemization with enzymatic resolution. It has been demonstrated that racemic alcohols, esters, and amines can be efficiently converted to the corresponding enantiomeric products through the enzyme–metal catalyzed DKR. In the DKR of alcohols, a pair of complementary procedures are now available for the preparation of both (*R*)- and (*S*)-products. The DKR, performed at room temperature with commercially available enzymes and metal catalyst, can be applicable to a wide range of substrates. However, the DKR of amines is limited to benzylic amines and requires high temperature. Accordingly, for the efficient DKR of amines, further efforts will be directed toward developing practical racemizing catalysts with high activity and broad specificity at room temperature [67].

REFERENCES

1. Sheldon, R.A., *Chirotechnology, Industrial Synthesis of Optically Active Compounds*, Marcel Dekker, New York, 1993.
2. Wong, C.-H. and Whitesides, G.M., *Enzymes in Synthetic Organic Chemistry*, Pergamon Press, Oxford, UK, 1994.
3. Koskinen, A.M.P. and Klivanov, A.M., Eds., *Enzymatic Reactions in Organic Media*, Blackie Academic & Professional, Glasgow, Scotland, 1996.
4. Faber, K., *Biotransformations in Organic Chemistry*, 3rd ed., Springer, Berlin, Germany, 1997.
5. Bornscheuer, U.T. and Kazlauskas, R.J., *Hydrolases in Organic Synthesis*, Wiley-VCH, Weinheim, Germany, 1999.
6. Drauz, K. and Waldmann, H., *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook*, 2nd ed., vols. I–III, Wiley-VCH, Weinheim, Germany, 2002.
7. Kim, M.-J., Choi, G.-B., Kim, J.-J., et al., Lipase-catalyzed transesterification as a practical route to homochiral acyclic *anti*-1,2-diol. A new synthesis of (+)- and (–)-*endo*-brevicomine, *Tetrahedron Lett.*, 36, 6253, 1995.
8. Kim, M.-J. and Lim, I.-T., Synthesis of unsaturated C8–C9 sugars by enzymatic chain elongation, *Synlett*, 2, 138, 1996.
9. Kim, M.-J., Lim, I.-T., Choi, G.-B., et al., The efficient resolution of protected diols and hydroxy aldehydes by lipases: steric auxiliary approach and synthetic applications, *Bioorg. Med. Chem. Lett.*, 6, 71, 1996.
10. Kim, M.-J., Lim, I.-T., Kim, H.-J., et al., Enzymatic single aldol reactions of remote dialdehydes, *Tetrahedron: Asymmetry*, 8, 1507, 1997.
11. Lee, D. and Kim, M.-J., Lipase-catalyzed transesterification as a practical route to homochiral *syn*-1,2-diols. The synthesis of the taxol side chain, *Tetrahedron Lett.*, 39, 2163, 1998.
12. Chung, S.-K., Chang, Y.-T., Lee, E.J., et al., Synthesis of two enantiomeric pairs of myo-inositol-(1,2,4,5,6) and myo-inositol-(1,2,3,4,5)pentakisphosphate, *Bioorg. Med. Chem. Lett.*, 8, 1503, 1998.
13. Lee, D., Kim, K.C., and Kim, M.-J., Selective enzymatic acylation of 10-deacetylbaicatin III, *Tetrahedron Lett.*, 39, 9039, 1998.
14. Lee, D. and Kim, M.-J., Enzymatic selective dehydration and skeleton rearrangement of paclitaxel precursors, *Org. Lett.*, 1, 925, 1999.

15. Im, A.S., Cheong, C.S., and Lee, S.H., Lipase-catalyzed remote kinetic resolution of quaternary carbon-containing alcohols and determination of their absolute configuration, *Bull. Korean Chem. Soc.*, 24, 1269, 2003.
16. Kang, H.-Y., Ji, Y., Yu, Y.-K., et al., Synthesis of α -ketobutyrolactones and γ -hydroxy- α -keto acids, *Bull. Korean Chem. Soc.*, 24, 1819, 2003.
17. Ward, R.S., Dynamic kinetic resolution, *Tetrahedron: Asymmetry*, 6, 1475, 1995.
18. Stürmer, R., Enzymes and transition metal complexes in tandem—a new concept for dynamic kinetic resolution, *Angew. Chem. Int. Ed. Engl.*, 36, 1173, 1997.
19. El Gihani, M.T. and Williams, J.M.J., Dynamic kinetic resolution, *Curr. Opin. Biotechnol.*, 3, 11, 1999.
20. Azerad, R. and Buisson, D., Dynamic resolution and stereoinversion of secondary alcohols by chemo-enzymatic processes, *Curr. Opin. Biotechnol.*, 11, 565, 2000.
21. Huerta, F.F., Minidis, A.B.E., and Bäckvall, J.-E., Racemisation in asymmetric synthesis. Dynamic kinetic resolution and related processes in enzyme and metal catalysis, *Chem. Soc. Rev.*, 30, 321, 2001.
22. Kim, M.-J., Ahn, Y., and Park, J., Dynamic kinetic resolutions and asymmetric transformations by enzymes coupled with metal catalysis, *J. Curr. Opin. Biotechnol.*, 13, 578, 2002.
23. Pellissier, H., Dynamic kinetic resolution, *Tetrahedron*, 59, 8291, 2003.
24. Pàmies, O. and Bäckvall, J.-E., Combination of enzymes and metal catalysts. A powerful approach in asymmetric catalysis, *Chem. Rev.*, 103, 3247, 2003.
25. Pàmies, O. and Bäckvall, J.-E., Combined metal catalysis and biocatalysis for an efficient deracemization process, *Curr. Opin. Biotechnol.*, 14, 407, 2003.
26. Turner, N.J., Enzyme catalysed deracemisation and dynamic kinetic resolution reactions, *Curr. Opin. Chem. Biol.*, 8, 114, 2004.
27. Allen, J.V. and Williams, J.M.J., Dynamic kinetic resolution with enzyme and palladium combinations, *Tetrahedron Lett.*, 37, 1859, 1996.
28. Dinh, P.M., Howarth, J.A., Hudnott, A.R., et al., Catalytic racemization of alcohols: applications to enzymatic resolution reactions, *Tetrahedron Lett.*, 37, 7623, 1996.
29. Reetz, M.T. and Schimossek, K., Lipase-catalyzed dynamic kinetic resolution of chiral amines: use of palladium as the racemization catalyst, *Chimia*, 50, 668, 1996.
30. Choi, J.H., Kim, Y.H., Nam, S.H., et al., Aminocyclopentadienyl ruthenium chloride: catalytic racemization and dynamic kinetic resolution of alcohols at ambient temperature, *Angew. Chem. Int. Ed. Engl.*, 41, 2373, 2002.
31. Larsson, A.L.E., Persson, B.A., and Bäckvall, J.-E., Enzymatic resolution of alcohols coupled with ruthenium-catalyzed racemization of the substrate alcohol, *Angew. Chem. Int. Ed. Engl.*, 36, 1211, 1997.
32. Persson, B.A., Larsson, A.L.E., Ray, M.L., and Bäckvall, J.-E., Ruthenium- and enzyme-catalyzed dynamic kinetic resolution of secondary alcohols, *J. Am. Chem. Soc.*, 121, 1645, 1999.
33. Lee, H.K. and Ahn, Y., Lipase/ruthenium-catalyzed dynamic kinetic resolution of β -hydroxyalkylferrocene derivatives, *Bull. Korean Chem. Soc.*, 25, 1471, 2004.
34. Koh, J.H., Jeong, H.M., Kim, M.-J., et al., Enzymatic resolution of secondary alcohols coupled with ruthenium-catalyzed racemization without hydrogen, *Tetrahedron Lett.*, 40, 6281, 1999.
35. Lee, D., Huh, E.A., Kim, M.-J., et al., Dynamic kinetic resolution of allylic alcohols mediated by ruthenium- and lipase-based catalysts, *Org. Lett.*, 2, 2377, 2000.
36. Kim, K.W., Song, B., Choi, M.Y., et al., Biocatalysis in ionic liquids: markedly enhanced enantioselectivity of lipase, *Org. Lett.*, 3, 1507, 2001.
37. Lee, J.K. and Kim, M.-J., Ionic liquid-coated enzyme for biocatalysis in organic solvent, *J. Org. Chem.*, 67, 6845, 2002.
38. Kim, M.-J., Choi, M.Y., Lee, J.K., et al., Enzymatic selective acylation of glycosides in ionic liquids: significantly enhanced reactivity and regioselectivity, *J. Mol. Catal. B. Enzym.*, 26, 115, 2003.
39. Erbdinger, M., Mesiano, A.J., and Russel, A., Enzymatic catalysis of formation of Z-aspartame in ionic liquid—an alternative to enzymatic catalysis in organic solvents, *Biotechnol. Prog.*, 16, 1129, 2000.

40. Lau, R.M., van Rantwijk, F., Seddon, K.R., et al., Lipase-catalyzed reactions in ionic liquids, *Org. Lett.*, 2, 4189, 2000.
41. Itoh, T., Akasaki, E., Kudo, K., et al., Lipase-catalyzed enantioselective acylation in the ionic liquid solvent system: reaction of enzyme anchored to the solvent, *Chem. Lett.*, 262, 2001.
42. Schoefer, S.H., Kraftzik, N., Wasserscheid, P., et al., Enzyme catalysis in ionic liquids: lipase catalysed kinetic resolution of 1-phenylethanol with improved enantioselectivity, *Chem. Commun.*, 425, 2001.
43. Park, S. and Kazlauskas, R., Improved preparation and use of room-temperature ionic liquids in lipase-catalyzed enantio- and regioselective acylations, *J. Org. Chem.*, 66, 8395, 2001.
44. Kim, M.-J., Kim, H.M., Kim, D., et al., Dynamic kinetic resolution of secondary alcohols by enzyme-metal combinations in ionic liquid, *Green Chem.*, 6, 471, 2004.
45. Choi, J.H., Choi, Y.K., Kim, Y.H., et al., Aminocyclopentadienyl ruthenium complexes as racemization catalysts for dynamic kinetic resolution of secondary alcohols at ambient temperature, *J. Org. Chem.*, 69, 1972, 2004.
46. Csajnyik, G., Bogár, K., Bäckvall, J.-E., New efficient ruthenium catalysts for racemization of alcohols at room temperature, *Tetrahedron Lett.*, 45, 6799, 2004.
47. Martin-Matute, B., Edin, M., Bogár, K., et al., Highly compatible metal and enzyme catalysts for efficient dynamic kinetic resolution of alcohols at ambient temperature, *Angew. Chem. Int. Ed. Engl.*, 43, 6535, 2004.
48. Martin-Matute, B., Edin, M., Bogár, K., et al., Combined ruthenium(II) and lipase catalysis for efficient dynamic kinetic resolution of secondary alcohols. Insight into the racemization mechanism, *J. Am. Chem. Soc.*, 127, 8817, 2005.
49. Dijkman, A., Elzinga, J.M., Li, Y.X., et al., Efficient ruthenium-catalyzed racemization of secondary alcohols: application to dynamic kinetic resolution, *Tetrahedron: Asymmetry*, 13, 879, 2002.
50. Persson, B.A., Huerta, F.F., and Bäckvall, J.-E., Dynamic kinetic resolution of secondary diols via coupled ruthenium and enzyme catalysis, *J. Org. Chem.*, 64, 5237, 1999.
51. Edin, M. and Bäckvall, J.-E., On the mechanism of the unexpected facile formation of meso-diacetate products in enzymatic acetylation of alkanediols, *J. Org. Chem.*, 68, 2216, 2003.
52. Huerta, F.F., Laxmi, S.Y.R., and Bäckvall, J.-E., Dynamic kinetic resolution of α -hydroxy acid esters, *Org. Lett.*, 2, 1037, 2000.
53. Runmo, A.B.L., Pàmies, O., Faber, K., et al., Dynamic kinetic resolution of γ -hydroxy acid derivatives, *Tetrahedron Lett.*, 43, 2983, 2002.
54. Pàmies, O. and Bäckvall, J.-E., Enzymatic kinetic resolution and chemoenzymatic dynamic kinetic resolution of δ -hydroxy esters. An efficient route to chiral δ -lactones, *J. Org. Chem.*, 67, 1261, 2002.
55. Pàmies, O. and Bäckvall, J.-E., Dynamic kinetic resolution of β -azido alcohols. An efficient route to chiral aziridines and β -amino alcohols, *J. Org. Chem.*, 66, 4022, 2001.
56. Kim, M.-J., Choi, Y.K., Choi, M.Y., et al., Lipase/ruthenium-catalyzed dynamic kinetic resolution of hydroxy acids, diols, and hydroxy aldehydes protected with a bulky group, *J. Org. Chem.*, 66, 4736, 2001.
57. Pàmies, O. and Bäckvall, J.-E., Efficient lipase-catalyzed kinetic resolution and dynamic kinetic resolution of β -hydroxy nitriles. A route to useful precursors for γ -amino alcohols, *Adv. Synth. Catal.*, 343, 726, 2001.
58. Pàmies, O. and Bäckvall, J.-E., Chemoenzymatic dynamic kinetic resolution of β -halo alcohols. An efficient route to chiral epoxides, *J. Org. Chem.*, 67, 9006, 2002.
59. Pàmies, O. and Bäckvall, J.-E., An efficient route to chiral α - and β -hydroxyalkanephosphonates, *J. Org. Chem.*, 68, 4815, 2003.
60. Kim, M.-J., Chung, Y.I., Choi, Y.K., et al., (*S*)-selective dynamic kinetic resolution of secondary alcohols by the combination of subtilisin and an aminocyclopentadienylruthenium complex as the catalysts, *J. Am. Chem. Soc.*, 125, 11494, 2003.
61. Choi, Y.K., Suh, J.H., Lee, D., et al., Dynamic kinetic resolution of acyclic allylic acetates using lipase and palladium, *J. Org. Chem.*, 64, 8423, 1999.
62. Pàmies, O., Ell, A.H., Samec, J.S.M., et al., An efficient and mild ruthenium-catalyzed racemization of amines: application to the synthesis of enantiomerically pure amines, *Tetrahedron Lett.*, 43, 4699, 2002.

63. Jung, H.M., Koh, J.H., Kim, M.-J., et al., Concerted catalytic reactions for conversion of ketones or enol acetates to chiral acetates, *Org. Lett.*, 2, 409, 2000.
64. Jung, H.M., Koh, J.H., Kim, M.-J., et al., Practical ruthenium/lipase-catalyzed asymmetric transformations of ketones and enol acetates to chiral acetates, *Org. Lett.*, 2, 2487, 2000.
65. Kim, M.-J., Choi, M.Y., Han, M.Y., et al., Asymmetric transformations of acyloxyphenyl ketones by enzyme-metal mult catalysis, *J. Org. Chem.*, 67, 9481, 2002.
66. Choi, Y.K., Kim, M.-J., Ahn, Y., et al., Lipase/palladium-catalyzed asymmetric transformations of ketoximes to optically active amines, *Org. Lett.*, 3, 4099, 2001.
67. Kim, W.-H., Karvembu, R., and Park, J., Alumina-supported ruthenium catalysts for the racemization of secondary alcohols, *Bull. Korean Chem. Soc.*, 25, 931, 2004.

9 Biotransformation of Natural or Synthetic Compounds for the Generation of Molecular Diversity

Robert Azerad

CONTENTS

9.1	Introduction	273
9.2	Flavonoid Derivatives	274
9.3	FK-506 and FK-520	280
9.4	Avermectins and Milbemycins	283
9.5	Miscellaneous Biotransformations	288
9.6	Conclusion	290
	References	291

9.1 INTRODUCTION

In the last decade, combinatorial chemistry has become a versatile and powerful tool for generating libraries of new chemical entities for the pharmaceutical industry. Meanwhile, biocatalytic methods have developed and provided complementary approaches and in some cases powerful alternatives to conventional synthetic chemical techniques, due to their high chemo-, regio-, and stereoselectivity. Moreover, the ability of enzyme or microbial catalysis to perform difficult chemical reactions, such as hydroxylations of nonactivated carbons, on structurally complex molecules, without the need of protection/deprotection steps of reactive functional groups, was recognized. That promoted new strategies for the use of biocatalysis as an additional tool for the multiple modification of synthetic or natural products [1], and the development of a biocombinatorial chemistry (“combinatorial biocatalysis”) for generating molecular diversity, as a novel approach in drug discovery and development [2–4].

True combinatorial methods with associated deconvolution strategies have been described using enzymes either in the preparation of combinatorial libraries or in the postsynthetic modification of combinatorial libraries to amplify their structural diversity [5,6].

Several classes of enzymic reactions have been used, sometimes in combined rounds [4] or in a one-pot reaction cascade: acylations with lipases [4,5,7], glycosylations [8], oxido-reductions by dehydrogenases [9,10], aldolase reactions [11], etc.

More examples are related to parallel syntheses, operating with a collection of microorganisms to produce either from a single substrate or a library of related substrates, a number of different derivatives, making use of the versatility of the microorganism-catalyzed biotransformations [12–14].

This chapter focuses on biooxidative transformations [15,16] (hydroxylations and dihydroxylations) of structurally complex natural or synthetic compounds of biological interest to generate in a parallel or combinatorial approach, known animal metabolites or novel derivatives exhibiting new or modified pharmacological or pharmacokinetic properties. The first accomplishment of this methodology came from the use of microorganism models to mimic the numerous animal or human detoxication reactions of drugs and xenobiotics [12], also shown to generate new bioactive molecules [17]. This concept has been widely applied in the fields of steroids [18–20], terpenoids [13,21], alkaloids [22], and synthetic chemicals [14,15] to functionalize these compounds and to generate new oxidized chemical entities. Cloned animal P450 monooxygenases [23–25] and cloned bacterial dioxygenases [26,27] have also been successfully employed to this end.

In contrast to combinatorial biocatalysis, “combinatorial biosynthesis” [28–32] makes use of genetic contribution and recent improvements in the understanding of natural biosynthetic pathways [33–35] to produce mutated or recombinant microorganisms and enzymes able to generate modified natural product analogs, in the same way as those structures have evolved over time in nature [36–38]. This has been demonstrated in the field of polyketide macrolides, e.g., with erythromycin, where a wide diversity of modified products have been generated by engineering every step of the modular biosynthetic megasynthase and associated reactions [39], including the introduction of a functional complete pathway in a heterologous host to overproduce these complex analogs [40–42].

Combinatorial biosynthesis (including mutational biosynthesis, precursor-directed biosynthesis, and cyclization pattern modifications) constitutes an important source for the future development of novel secondary metabolite structures and for the generation of structural diversity in natural product libraries. Several examples of this promising approach will be presented in addition to the use of classical biotransformation studies, between two classes of natural polyketides: FK-506/FK-520 and avermectin/milbemycin.

9.2 FLAVONOID DERIVATIVES

Flavonoids represent a very interesting group of ubiquitous plant secondary metabolites. They have been shown to have a wide range of beneficial pharmacological and health effects generally related to their antioxidant properties. Based on their backbone structure (Figure 9.1), they are grouped into several major classes that include substituted flavones **1**, flavanones **2**, isoflavones **3**, chalcones **4**, etc. from which an enormous variety of structures is derived by modification of the backbone substituents.

Although 6500 flavonoids have so far been identified in the *Handbook of Natural Flavonoids* [43], biological transformation of these compounds has been a matter of relatively few studies. In addition to chemical synthesis, it may be a valuable approach to increase their

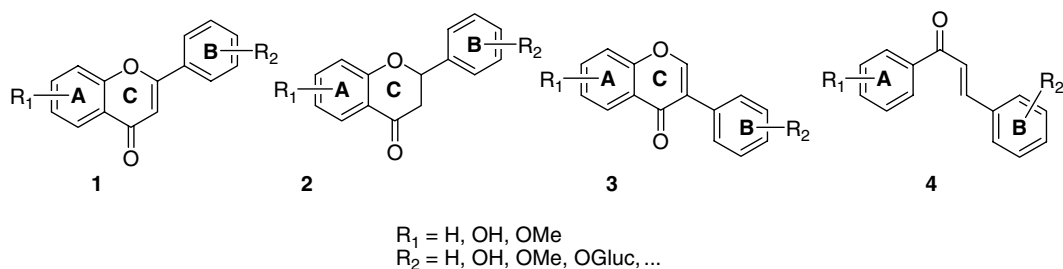


FIGURE 9.1 Main backbones and families of flavonoid compounds.

natural diversity and produce unnatural and potentially active derivatives by enzymatic or microbial conversion.

Enzymatic transformations of flavonoids have been essentially directed to the regioselective glycosylation of the phenolic groups and the subsequent acylation of the resulting glycosides. Several glycosidases readily accept flavonoids as substrates to prepare regioselectively glycosylated derivatives in moderate yields [44]. The resulting flavonoid glycosides were then subsequently modified, using classical lipase or esterase reactions with various activated aromatic or aliphatic ester groups [45–47] to obtain regioselectively acylated derivatives mimicking natural compounds of particular interest due to their intrinsic biological activities.

Other transformations are mainly concerned with oxidative reactions of flavonoid compounds, either oxidative coupling or additional introduction of hydroxyl groups.

For example, theaflavins are formed by fermentative oxidation of catechin in black tea and share with other polyphenols of tea a considerable interest because of their potential benefits for human health and reduction in the risk of cardiovascular diseases and cancer. In an attempt to prepare significant amounts of similar theaflavin derivatives, 18 different related compounds have been obtained using coupling reactions of selected pairs of flavonoid reactants catalyzed by the horseradish peroxidase/hydrogen peroxide system [48], as shown in Figure 9.2. The reaction involves the oxidation of the B-rings to quinones, followed by the

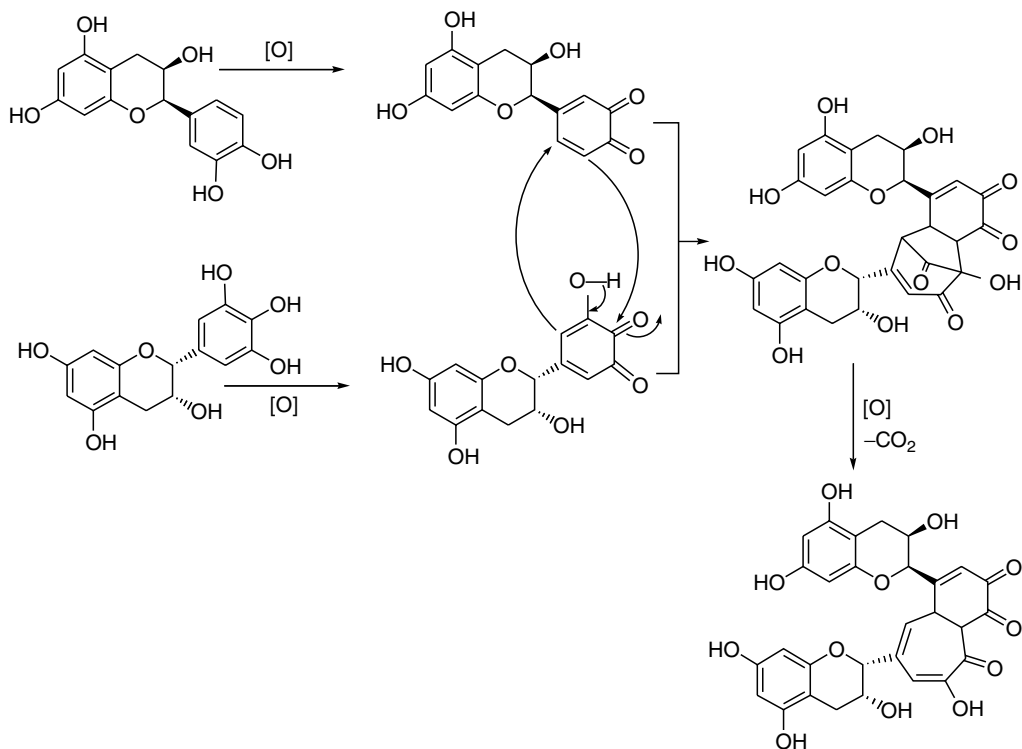


FIGURE 9.2 Hydrogen peroxide/horseradish peroxidase-catalyzed oxidation of a couple of flavanoid moieties to a theaflavin-like derivative. (Adapted from Sang, S., Lambert, J.D., Tian, S., et al., *Bioorg. Med. Chem.*, 12, 459–467, 2004.)

Michael addition of the gallo catechin quinone to the catechin quinone prior to the carbonyl addition across the ring and subsequent decarboxylation.

Most of the microbial (whole-cell) transformations of flavonoids have been found to occur in rings A and B, particularly at C-5 to C-7, C-4' and to a lesser extent at C-8 or C-3' [49,50]. Such transformations essentially include monohydroxylation [51–54], *O*-demethylation [52,55–57] or *O*-methylation [58,59], and less frequently C-methylation [59] or conjugation [55,60–62]. Characteristic examples of these reactions using various fungi or *Streptomyces* strains are shown in Figure 9.3 and Figure 9.4, respectively. Some of them are exact mimics of the animal or human metabolism of dietary flavonoids by dedicated liver CYP450s [63–67]. Bacterial strains isolated from intestinal tract [68,69] and fermented foods or drinks [70] have also been used for similar transformations.

A peculiar reaction has been described for prenylated isoflavone compounds, such as 7-*O*-methyl luteone [71–73], which is cyclized by *Botrytis cinerea* to the corresponding dihydrofurano- and dihydropyrano-fused derivatives upon an initial monooxygenase-catalyzed epoxidation of the prenyl side chain (Figure 9.5).

There have been only a few reports concerning the microbial transformation of chalcones 4. In addition to hydroxylation or *O*-demethylation reactions, a strain of *Aspergillus alliaceus*

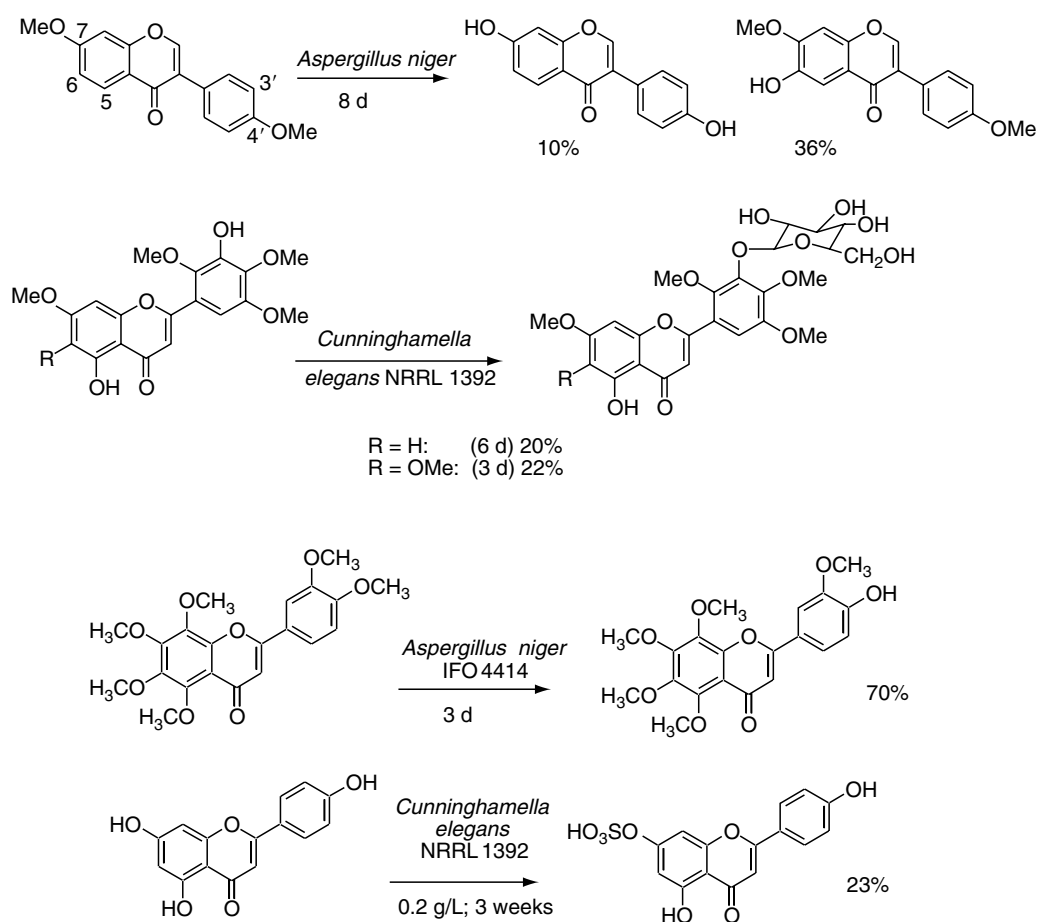


FIGURE 9.3 Some biotransformations of natural flavonoid compounds by fungi. (From Okuno, Y. and Miyazawa, M., *J. Nat. Prod.*, 67, 1876–1878, 2004; Ibrahim, A.R., *Phytochemistry*, 53, 209–212, 2000.)

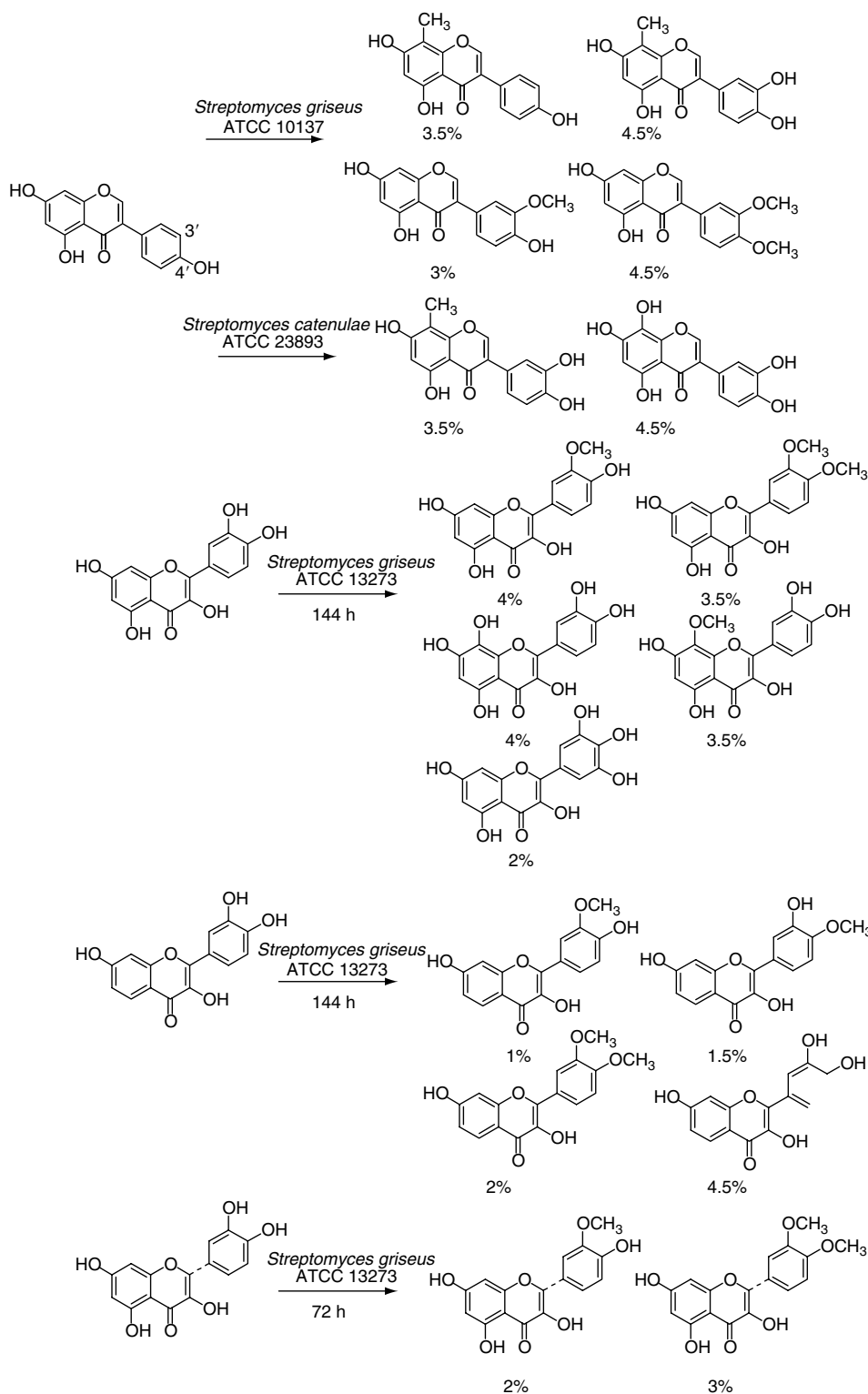


FIGURE 9.4 Some biotransformations of natural flavonoid compounds by bacteria. (From Hosny, M., Dhar, K., and Rosazza, J.P.N., *J. Nat. Prod.*, 64, 462–465, 2001; Hosny, M. and Rosazza, J.P.N., *J. Nat. Prod.*, 62, 1609–1612, 1999; Klus, K. and Barz, W., *Phytochemistry*, 47, 1045–1048, 1998.)

(continued)

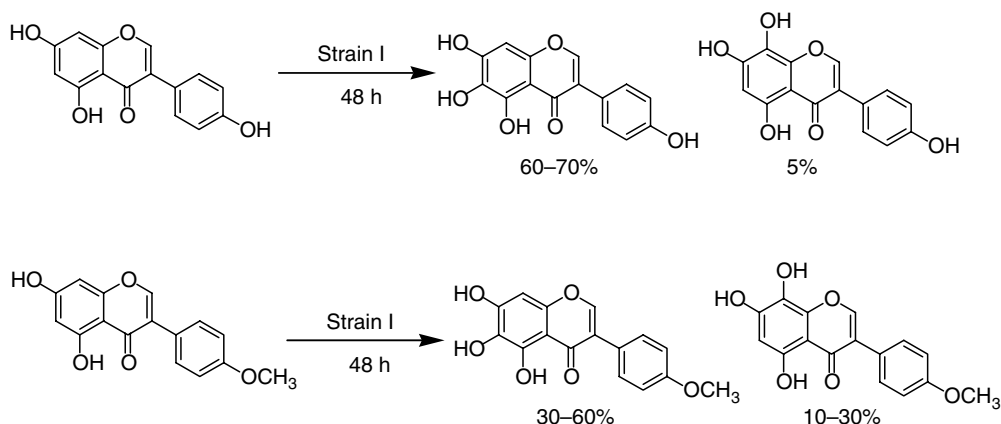


FIGURE 9.4 (continued)

was reported to cyclize chalcones to flavanones [74], mimicking a plant biosynthetic process (Figure 9.6). However, the flavonoid products are racemic, unlike the corresponding stereoselectively cyclized compounds found in plants.

The antioxidant activity of natural flavonoid compounds, considered to be of medicinal and nutritional importance, is strongly dependent on the presence of an *o*-dihydroxylated structure in the B-ring. In an attempt to increase molecular diversity and antioxidative activity of natural or synthetic compounds, genetically engineered dioxygenase systems such as recombinant microbial 2,3-biphenyl dioxygenases have been recently developed to generate *o*-dihydroxylated phenyl derivatives [75] from the corresponding natural or synthetic flavonoids.

Streptomyces lividans whole-cells expressing a shuffled broad-specificity large unit of the biphenyl dioxygenase gene *bphA1* (2072) derived from *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia cepacia* LB400, in combination with *bphA2* (small subunit of the iron–sulfur protein), *bphA3* (ferredoxin), and *bphA4* (ferredoxin reductase) transform in moderate yields flavone, flavanone, 6-hydroxyflavanone, and 6-hydroxyflavone into corresponding *o*-dihydroxylated or monohydroxylated derivatives in various positions of the phenyl (B-ring) group [76].

Similar results, with a prominent conversion to 2',3'- or 3',4'-catechol derivatives have been recently reported using an *Escherichia coli* strain expressing the same dioxygenase

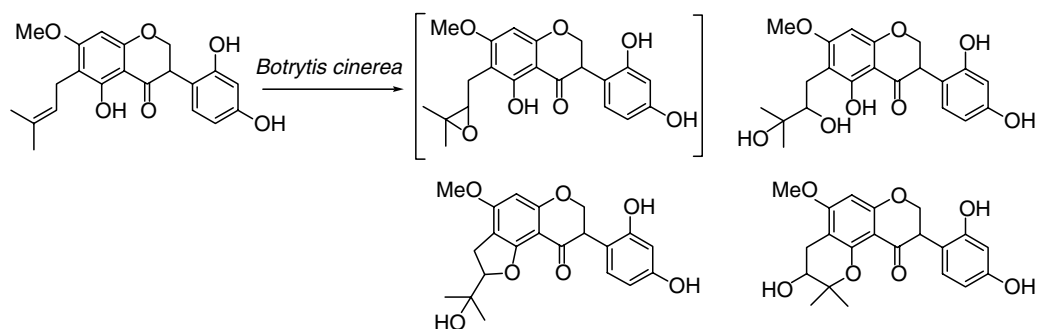


FIGURE 9.5 Biotransformation of 7-*O*-methyl luteone by whole-cells or enzymic extract of *Botrytis cinerea*.

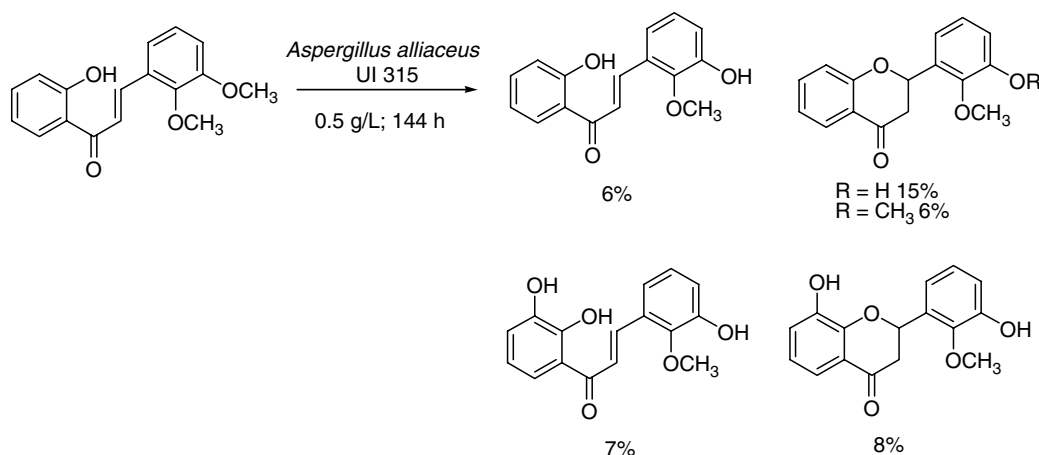
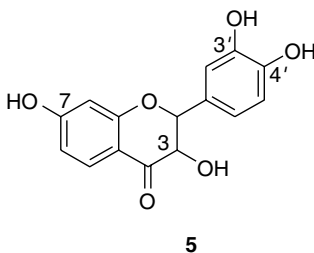


FIGURE 9.6 Biotransformation of a chalcone by a strain of *Aspergillus alliaceus*. (From Sanchez-Gonzalez, M. and Rosazza, J.P.N., *J. Nat. Prod.*, 67, 553–558, 2004.)

system, in addition to the dihydrodiol dehydrogenase gene *bhpB* from *P. pseudoalcaligenes* KF707 [77]. By this method, “unnatural” flavonoids bearing a catechol structure in unusual positions were generated (Figure 9.7), which have not been previously detected in nature and which showed stronger antioxidant activities than their flavonoid natural precursors.

Other results dealing with the introduction of new hydroxy groups into isoflavonoid compounds (including halogenated compounds) using recombinant microbial enzymes have been independently reported by a Chilean group [78]. Recombinant whole-cells of *E. coli* similarly expressing biphenyl-2,3-dioxygenase (BphA) with or without the associated biphenyl-2,3-dihydrodiol 2,3-dehydrogenase (BphB) of *Burkholderia* sp. LB400 were used to generate, from fourteen synthetic isoflavonoids, 2',3'- and 3'-4'-dihydrodiols, plus the corresponding monohydroxylated compounds at the 2'-, 3'- and/or 4'-positions, resulting from a nonenzymatic dehydration in the absence of the dehydrogenase. Recombinant cells containing both BphA and BphB produced, sometimes in high yields, the corresponding catechol compounds, besides some monohydroxylated derivatives (Figure 9.8).

Another example of the diversification of flavonoid structures by recombinant enzymic methods has been described [79], using several plant flavonoid-modifying enzymes with different substrate specificities, namely an *O*-glucosyltransferase (GT) from *Arabidopsis thaliana* and several *O*-methyltransferases (OMTs) from *Mentha piperita*, expressed in *E. coli*. Single or mixed recombinant cultures were tested for *O*-glucosidation and *O*-methylation of quercetin **5** as a model substrate for the generation of regioselective derivatives.



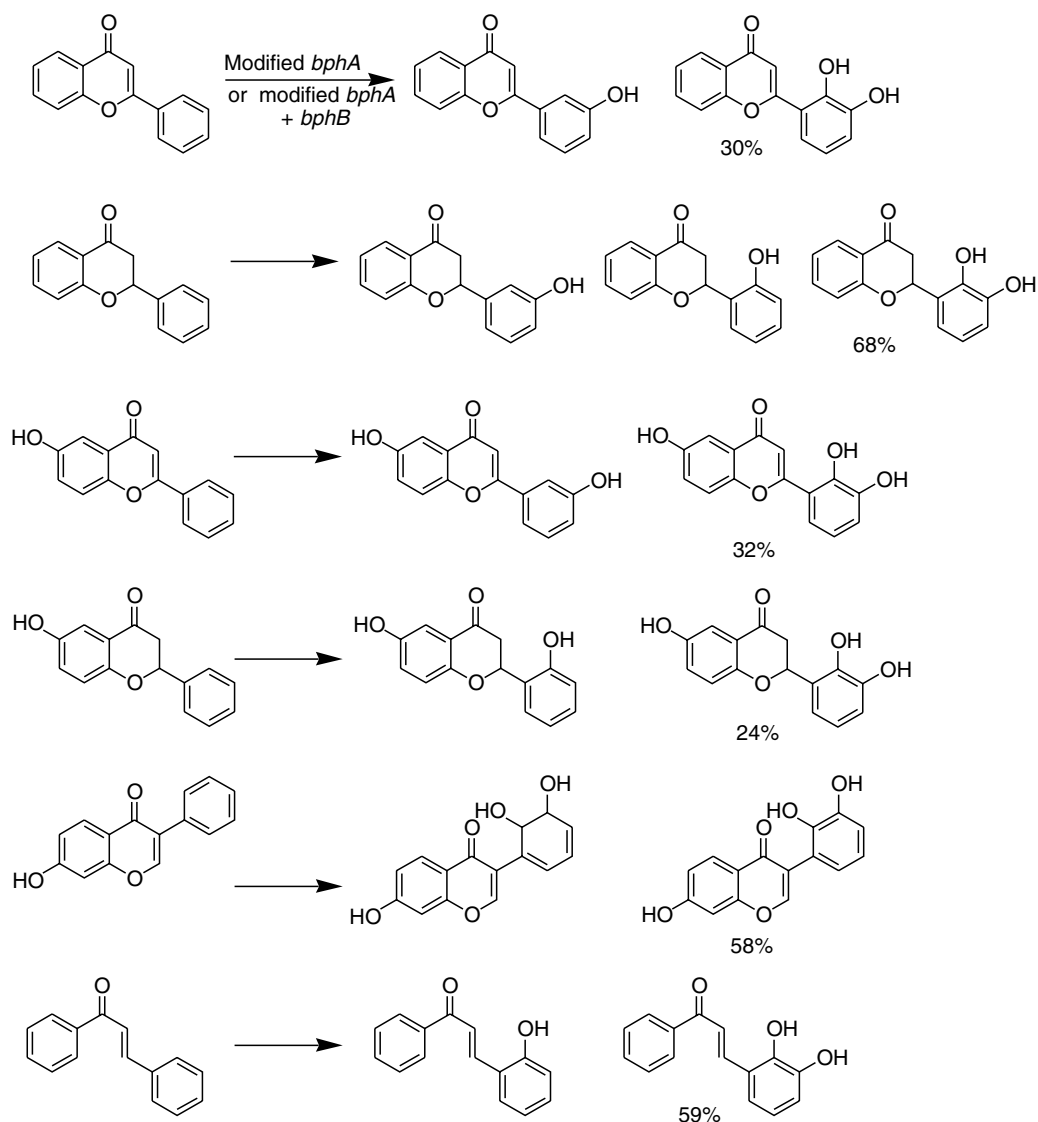


FIGURE 9.7 Hydroxylated derivatives generated from simple flavonoids by whole-cells of *E. coli* expressing shuffled BphA (2,3-biphenyl dioxygenase) and BphB (2,3-dihydrodiol dehydrogenase).

When induced cultures expressing each of the individual genes were supplemented with quercetin (up to 0.3 mM), regiospecific 7-, 3'-, or 4'-*O*-methylated and 7-*O*-glucosylated derivatives were formed in 25 to 50% yields, in agreement with previous *in vitro* studies with purified enzymes. When combinatorial mixed-culture experiments were performed involving several OMTs and/or GT, a variety of the expected partially and completely modified flavonoids were obtained, indicating the feasibility of a simultaneous expression of the different enzyme classes.

9.3 FK-506 AND FK-520

FK-506 (**6**, tacrolimus, L-679,934) and FK-520 (**7**, ascomycin, immunomycin, L-683,590), two immunosuppressive macrolide compounds, respectively isolated from *S. tsukubaensis*

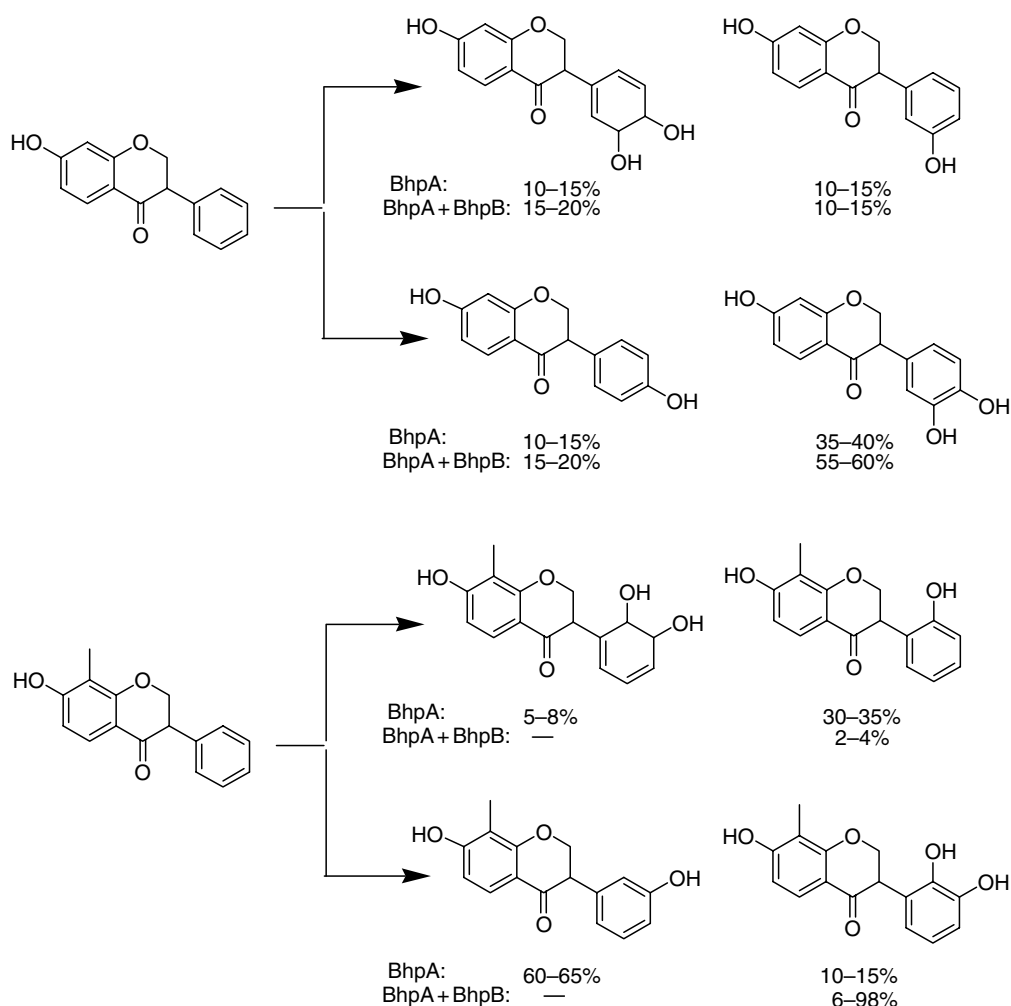


FIGURE 9.8 Hydroxylated derivatives generated from two synthetic isoflavonoids by whole-cells of *E. coli* expressing BhpA (2,3-biphenyl dioxygenase) and BhpB (2,3-dihydrodiol dehydrogenase).

[80,81] and *S. hygroscopicus* subsp. *yakushimaensis* No. 7238 [82] or var. *ascomyceticus* [83], have been developed as highly effective therapeutic agents in human organ transplants. Due to undesirable side effects and limited bioavailability, both molecules have been the subject of an extensive screening program at the Merck Research Laboratories for their structural modification and for the generation of new active derivatives. Due to the complexity of these molecules, mainly biological approaches were contemplated and a screening for microbial transformations was initiated [84]. A series of actinomycetes were isolated, which were able to perform regioselective hydroxylation or *O*-demethylation reactions of the natural compounds **6–7** or the semisynthetic derivatives **8–9**. A summary of the main biotransformations observed and effectively used for the preparation of their respective derivatives is shown in Table 9.1.

Regioselective single or combined *O*-demethylations in positions –13, –15, or –31 were prominent reactions, sometimes accompanied by rearrangement or cyclization reactions [85–94]. The 13-*O*-desmethyl derivative of **6** was previously isolated as its major metabolite in liver microsomal incubations [95,96].

TABLE 9.1
Some Microbial Transformations of FK-506 (6), FK-520 (7), and Semisynthetic Derivatives 8–9

<p>Chemical structure of FK-506 (6), a complex macrolide with multiple stereocenters and functional groups including hydroxyl, methoxy, and a piperidine ring.</p>	<p><i>Streptomyces rimosus</i> (MA187)</p>	<p>31-desmethyl FK-506</p>	<p>Ref.</p>
	<p><i>Actinomycete</i> sp. (MA6474) ATCC 53828</p>	<p>13-desmethyl rearranged (= L-683,519)</p>	<p>[89]</p>
			<p>[148]</p>
<p>Chemical structure of FK-520 (7), similar to FK-506 but with a different side chain at C-19.</p>	<p><i>Actinoplanes</i> sp. (MA6474) ATCC 53828</p>	<p>13-desmethyl rearranged (= L-685,487)</p>	<p>[91]</p>
	<p><i>Streptomyces</i> sp. (MA6870) ATCC 55281</p>	<p>Hydroxylation at C-19 methyl group and hemiketal formation with C-22 carbonyl</p>	<p>[99]</p>
	<p><i>Actinoplanes</i> sp. (MA6559) ATCC 53771</p>	<p>31-desmethylinmunomycin (= L-683,742)</p>	<p>[92]</p>
	<p><i>Streptomyces lavandulae</i> ATCC 55209</p>	<p>31- and 13-desmethyl rearranged (= L-683,756)</p>	<p>[149]</p>
		<p>31-desmethylation + hydroxylation at C-19 methyl group and hemiketal formation with C-22 carbonyl</p>	<p>[150, 151]</p>
<p>Chemical structure of semisynthetic derivative 8, featuring a 6-alkoxy-15-desmethyl ascomycin moiety linked to a piperidine ring.</p>	<p><i>Streptomyces lydicus</i> (?) (MA6890) ATCC 55387</p>	<p>13-desmethyl rearranged</p>	<p>[88]</p>
<p>Chemical structure of semisynthetic derivative 9, similar to 8 but with a different R group at the 6-position.</p>	<p><i>Actinoplanes</i> sp. ATCC 53771</p>	<p>6-alkoxy-15-desmethyl ascomycin</p>	<p>[94]</p>
<p>9, R =Et, Pr</p>			

However, none of the microorganisms screened was able to produce the 15-*O*-desmethylated derivative of **7**. An elegant solution for this problem was found by using the *Actinoplanes* sp. strain ATCC 53771 to produce the 15,31-*O,O*-bisdesmethyl derivative, then transforming it with the 31-*O*-methyltransferase isolated from *S. ascomyceticus* [90,97], which catalyzes the specific methylation of the hydroxyl group at position –31 of **7** [98]

Another reaction catalyzed by a different *Streptomyces* strain was an allylic hydroxylation of the methyl group at C-19, followed by a cyclic hemiketal formation with the C-22 carbonyl group [94,99].

Using modified culture media, the conversion of FK-520 to its 31-*O*-desmethyl derivative by *Actinoplanes* sp. ATCC 53771 could be improved from 15% to 35–45% yield to produce the desmethyl derivative in a 900 L-scale fermentation [94]. Besides the expected *O*-desmethyl derivative (18 g from 90 g FK-520), small amounts of the 31-*O*-desmethyl *epi* derivative (0.08 g) and of the 31-*O*-desmethyl-19(22)-hydroxymethyl ketal (0.64 g) were isolated.

Alternatively, the bioavailability of these highly lipophilic molecules could be increased by *O*-glucosylation at position –24, using a bioconversion with a *Bacillus subtilis* strain [100], or by *O*-phosphorylation at position –32, carried out by *Rhizopus oryzae* ATCC 11145 [84].

Recently, different approaches to the synthesis of new modified derivatives of FK-506 or FK-520 were undertaken by genetic engineering methods, following the nearly complete elucidation of the biosynthetic pathway of these molecules catalyzed by polyketide synthase (PKS) clusters in combination with post-PKS reactions [101,102].

One of these approaches was the disruption of a few genes, particularly those responsible for the post-PKS reactions, such the one involved in the hydroxylation of position –9, which led to the 9-deoxo or the 9-deoxo-31-*O*-desmethyl derivatives [103].

Another more sophisticated approach (“combinatorial biosynthesis”) was the replacement of selected acetyltransferase genes of the specific PKS clusters, by heterologous genes controlling the incorporation of new different acyl units in the sequential elaboration of the FK-506 or FK-520 skeleton, to synthesize novel unnatural polyketides [28,29]. The FK-506 or the FK-520 modular polyketide synthases (Type I) contain 10 modules [35,102] that catalyze sequential additions of two-, three- or four-carbon units to a shikimate-derived starter unit to form the polyketide chain (Figure 9.9). The chain is cyclized after a subsequent addition of pipercolic acid. Each module contains an acyltransferase that selects the particular acylCoA precursor for each round of chain extension.

The replacement of the ascomycin acyltransferase domains (AT7 and AT8) specific for the incorporation of putative methoxymalonate units [104] resulting in the formation of the 13- and 15-methoxy groups, by a malonyl, methylmalonyl, or ethylmalonyl heterologous acyltransferase domains from the PKS clusters of FK-520 or Rapamycin (a closely related structural analog), allowed the production of 13-desmethoxy-, 13,15-bisdesmethoxy- and various analogs methyl- or ethyl-substituted at positions –13 or –15 [104,105]. Other chimeric PKS with preprogrammed substrate specificity, in which biosynthetic modules are truncated, exchanged, or repositioned, should lead to the preparation of novel modified polyketide compounds, with potentially different biological profiles.

9.4 AVERMECTINS AND MILBEMYCINS

Avermectins (**10**) are a mixture of eight closely related 16-membered macrocyclic lactones isolated as fermentation products from *S. avermitilis* ATCC 31267 cultures (Figure 9.10). They contain at C-13, a disaccharide of the unusual methylated deoxysugar L-oleandrose and their diversity results from structural differences at C-5, C-22,23 and a variable side chain at C-25. Avermectins, the semisynthetic 22,23-dihydroavermectins **B** called ivermectins (**11**), and several of their derivatives are widely used for the treatment of animal diseases caused by

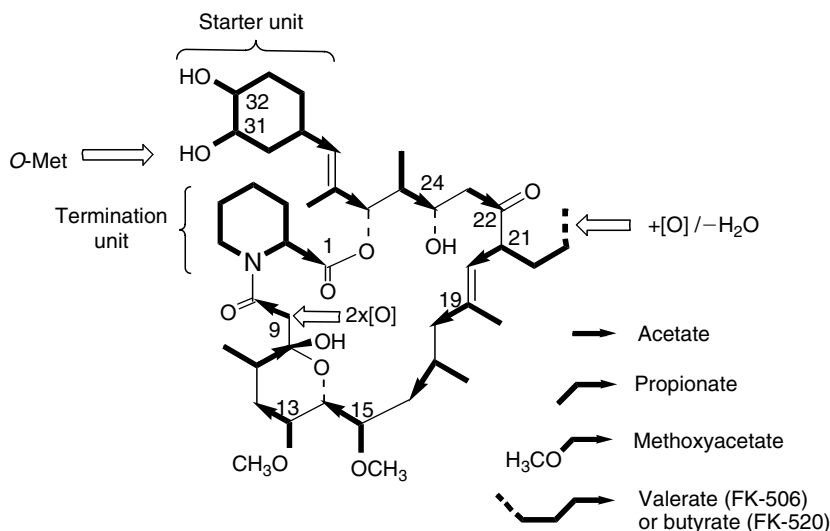


FIGURE 9.9 The biosynthetic pattern of FK-506/FK-520. The biosynthetic units resulting from the sequential acylCoA additions are indicated by bold arrows. Post-PKS modifications and their target units are indicated with empty arrows.

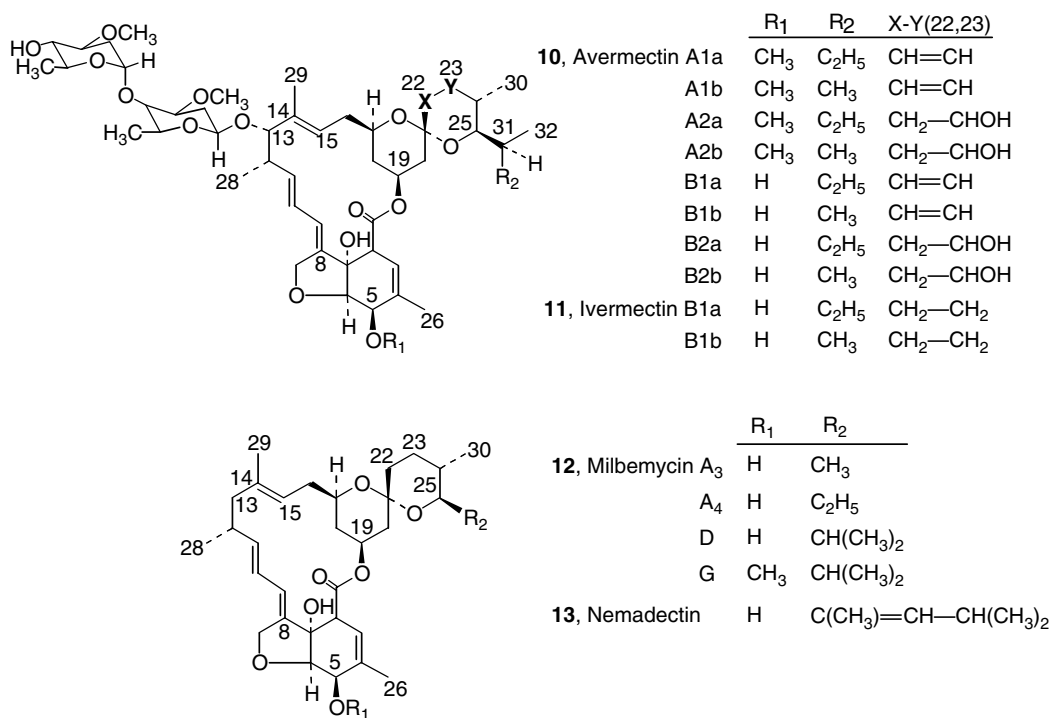


FIGURE 9.10 The main natural or semisynthetic components of the avermectin and milbemycin families.

parasitic nematodes and arthropods, and also for crop protection since 1980–1985. Ivermectins have recently been shown to be highly effective in treating human tropical parasitic diseases caused by microfilariae, such as onchocerciasis (river blindness) and strongyloidiasis.

Milbemycins (**12**), a closely related family of compounds produced by various *S. hygroscopicus* strains, lack the disaccharide unit at C-13 and differ from the 22,23-dihydroavermectin aglycones by their substituents at C-25 (Figure 9.10). Another related compound, Nemadectin (**13**) is produced by *S. thermoachaensis* or *S. cyaneogriseus*. They all similarly exhibit a broad spectrum of antiparasitic (anthelmintic), acaricidal, and insecticidal activities.

To obtain new derivatives for subsequent use as metabolite reference standards in animal metabolism studies, and to functionalize intermediates for subsequent site-directed chemical modifications, the microbial transformation of some of these natural products was extensively investigated by several industrial groups (Ramos Tombo et al. from CIBA-Geigy, Basel; Nakagawa et al. from Sankyo Ltd., Tokyo; and Arison et al. from Merck & Co, Rahway).

Results have been obtained using incubations of milbemycin compounds with various actinomycetes or fungi cultures, as shown with Milbemycin A4 (Figure 9.11). The isolated derivatives were formed by epoxidation at C-14,15 [106]; by hydroxylation at methyl groups located in external (mainly) allylic positions, C-26 [107], -28 [108,109], -29 [106,110], -30 [109,111,112], or in the C-25 side chain (C-31, C-32 [112]); and by hydroxylation at methylene groups involved in the carbon ring, such as C-24 [109] or C-13 [106,109,113]. The last one has been particularly useful in giving access to the more active avermectin (or ivermectin) family through various enzymatic, microbial, or chemical glycosylation reactions [114,115].

Acaricidal activity of milbemycins depends on the substituents at C-25 position. Oxidations on this side chain are difficult to obtain by chemical transformations, and the described microbial oxidations of the side chain at this position have been used as a preliminary functionalization method to prepare novel C-25 substituted Milbemycin A4 derivatives, with noticeably increased acaricidal activity [116].

Similarly, biotransformations have been described on avermectins, ivermectins or their aglycones to give reactive sites for directed chemical modifications. Diastereomeric side chain-hydroxylated derivatives have been obtained in a scaled-up process from Avermectin A1a [117–119] or Ivermectin B1a aglycone [120,121] (Figure 9.12).

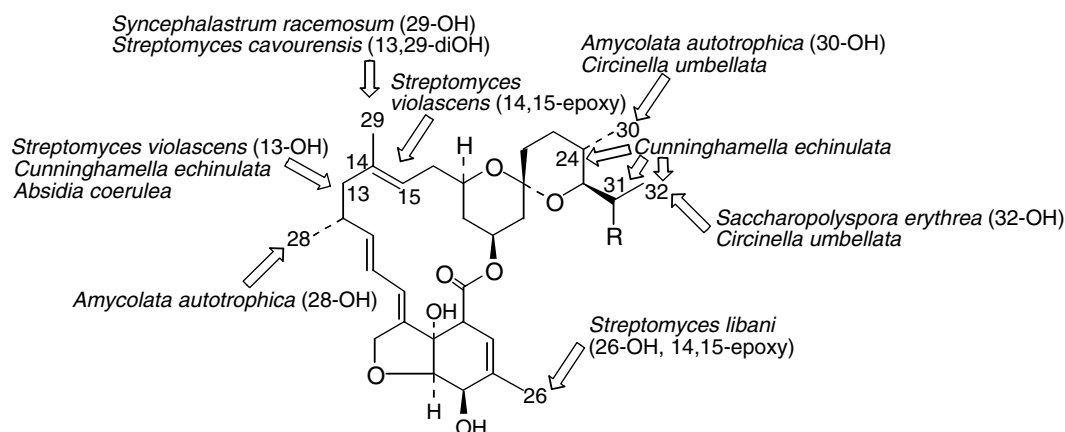


FIGURE 9.11 Some typical oxidative microbial transformations of milbemycins (milbemycin A4, R = H).

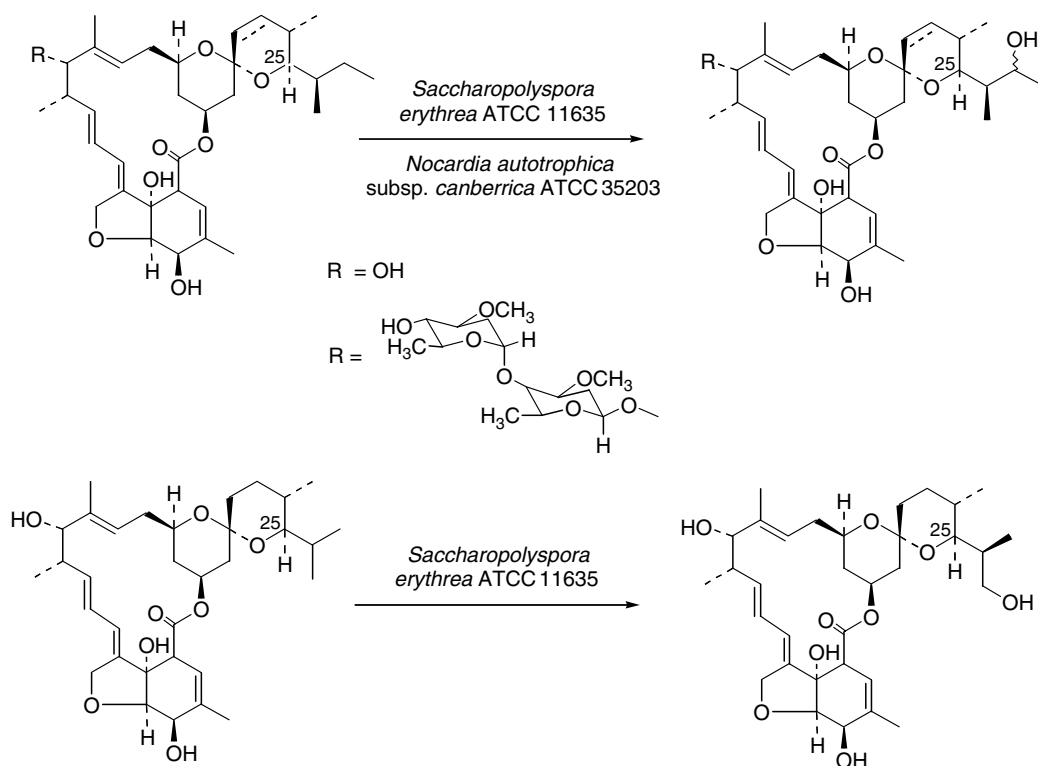


FIGURE 9.12 Microbial conversions of Avermectin A1a or ivermectin aglycones leading to hydroxylations on the C-25 side chain.

The cloning of the gene cluster for avermectin biosynthesis has been reported independently by the Merck group [122] and the Kitasato Institute group [123–125]. The knowledge of the complete genome sequence of *S. avermitilis* [126,127] and the progressive elucidation of the biosynthetic pathway of avermectins, still not achieved, have led to rational approaches to the selective production of desired components, or the production of novel avermectin derivatives, by “engineered biosynthesis,” through mutagenesis or recombinant DNA technologies.

The avermectin polyketide backbone is derived by sequential elongation from seven acetate and five propionate units (Figure 9.13) added to an α -branched chain fatty acid starter arising from the branched amino acid degradation pathway and corresponding to the C-25 substituent (*S*- α -methyl propionyl or isopropyl residues for “a” type or “b” type avermectins respectively). Postpolyketide synthase reactions are directed to dehydration at C-22,23 which modulates the B1:B2 ratio, monooxygenase reactions at C-6 and at C-8 methyl groups inducing the furane ring closure followed by the C-5 carbonyl reduction, *O*-methylation by *S*-adenosylmethionine, and stepwise introduction of the oleandrose disaccharide unit [128].

The main objects of these engineered biosynthetic approaches have been directed to the replacement of the C-25 side chain, to the modification of the C-5 substitution, and to the modification of the ratio in the biosynthesized product mixtures to favor the most active ones.

Avermectin homologs with longer side chains at C-25 are produced by *S. avermitilis* when cultures are supplied with high concentrations of 2-methylpentanoate or 2-methylhexanoate

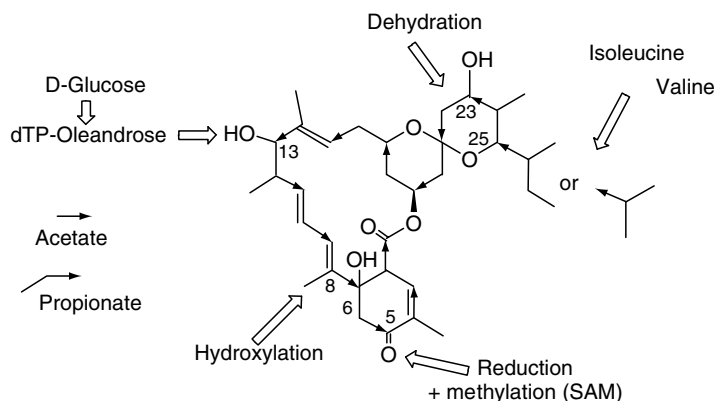


FIGURE 9.13 Incorporation of acetate and propionate units into the avermectin backbone. Post-PKS reactions are indicated with their target sites. (Adapted from Ikeda, H., Ishikawa, J., Hanamoto, A., et al., *Nat. Biotechnol.*, 21, 526–531, 2003.)

[129], indicating the ability of this species to incorporate new fatty acidCoAs as starter units for the polyketide synthase. Alternatively, *S. avermitilis* mutants that contain no functional branched chain 2-oxoacid dehydrogenase and are unable to grow with isoleucine, valine, and leucine as sole carbon sources have been isolated [130]. These mutants could synthesize avermectins when supplemented with exogenous α -branched chain fatty acids. Moreover, such mutants have been shown by the Pfizer group to form a corresponding series of novel avermectins when supplemented with a wide variety of fatty acids [131,132]. Noticeably, supplementation with cyclohexanecarboxylic acid (CHC) or introduction of a pathway that provided CHCCoA into the mutant [133] led to a C-25 cyclohexyl-substituted avermectin mixture CHC-B1 (Doramectin) and CHC-B2 (Figure 9.14). Engineering of *AveC*, a gene with undetermined mechanistic function, by DNA shuffling and extensive variant screening, has led to a considerably improved ratio of the more active Doramectin (CHC-B1) over the undesirable CHC-B2 component (1:0.07 vs. 1:1 for the parent strain), thus allowing a large scale development for the production of Doramectin [134].

The 5-keto compounds produced by *AveF* mutants or engineered strains result from a complete synthesis, lacking the non-PKS dependent reduction at this position [123,135]. As the yield of the corresponding selective chemical oxidation of the 5-hydroxyl group of

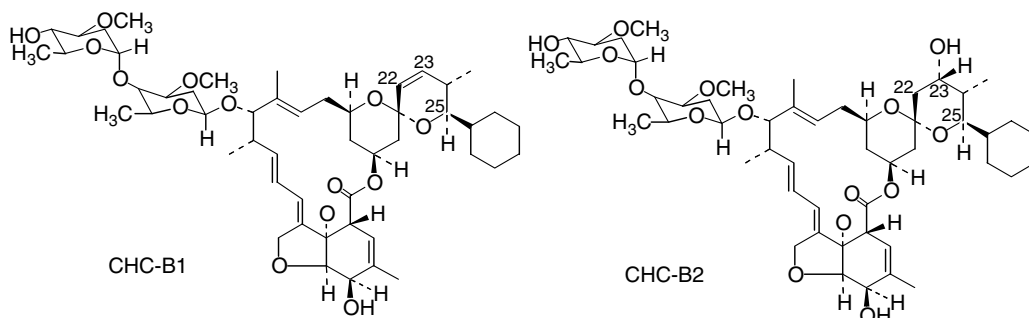


FIGURE 9.14 The main 25-cyclohexyl analogs of avermectins: CHC-B1 (Doramectin) and CHC-B2.

avermectin B components is low, the biosynthetic 5-oxo compounds thus obtained play an important role in the combinatorial biosynthesis of avermectins (and milbemycins), through their chemical conversion to very active 5-oxime derivatives [136].

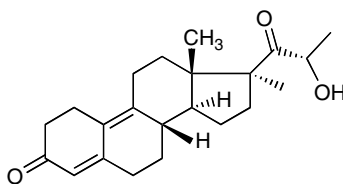
On the other hand, conversion of avermectins having a free hydroxyl group at C-5 (“B” component) to their *O*-methylated counterpart (“A” component) is catalyzed by a 5-methyl-*O*-transferase depending on the *aveD* gene [124,135]. Since the anthelmintic activity of “B” components is superior to that of “A,” mutations that cause the inactivation or the modulation of the methyltransferase are important for the production of more active avermectin mixtures.

As expected, the pentacyclic ring of milbemycins is also derived from a polyketide biosynthesis involving seven acetate and five propionate units. However, the milbemycin (and Nemadectin) PKS should contain a functional dehydratase and an additional enoylreductase in their tenth synthetic module to generate a fully saturated chain at C-13–14. Similarly, the presence of a saturated carbon chain at C-22,23, which is absent in natural avermectins, is explained by fully active enoyl reductase and dehydratase domains in the second synthetic module [123].

Exchange or complementation of PKS domains between *S. avermitilis* and milbemycin or other polyketide producing strains, if normally processed, should provide new opportunities for combinatorial biosynthesis of novel components, including the direct production of 22,23-dihydroavermectins [137]. As an example, the replacement of the original dehydratase of module 2 of avermectin synthetase in *S. avermitilis* by the functional dehydratase from the erythromycin eryAII module 4 by homologous recombination resulted in a recombinant strain producing only C-22,23-unsaturated avermectin compounds [138]. Concerning the post-PKS reactions, it has been demonstrated that the gene product corresponding to avermectin 5-*O*-methyl transferase from *S. avermitilis* is able to methylate at C-5 a milbemycin substrate with high efficiency [139].

9.5 MISCELLANEOUS BIOTRANSFORMATIONS

In an attempt to prepare the human metabolites of Trimegestone (**14**, RU27987), a second-generation progestomimetic steroid developed by Hoechst-Marion-Roussel in 1990–1995, and particularly, to obtain in sizable amounts minor metabolites of unknown structure observed in healthy volunteers, the parent compound was submitted to an extensive biotransformation screening using fungal and actinomycetal strains [18,140].



14

Nine oxidized derivatives (Table 9.2) were obtained in substantial yields, some of them as a single or a major product depending on the microorganism, allowing an easy preparation of each derivative in the 20 to 100 mg scale, a sufficient amount for complete identification and *in vitro* and *in vivo* assays. Among the new compounds obtained, two of them (1 β -hydroxy and 6 β -hydroxy derivatives) were identified as the unknown human metabolites previously

TABLE 9.2
Biotransformation of Trimegestone by Microorganisms

Metabolite Structure	Strain	Incubation Time (h)	Yield (%)
	<i>Absidia cylindrospora</i> LCP57.1569	24	20
	<i>Curvularia lunata</i> NRRL2380	24	70
	<i>Mucor hiemalis</i>	72	63
	<i>Absidia cylindrospora</i> LCP57.1569	24	10
	<i>Circinella minor</i>	72	23
	<i>Absidia corymbifera</i> LCP63.1800	168	34
	<i>Cunninghamella elegans</i> ATCC 26269	48	20
	<i>Absidia cylindrospora</i> LCP57.1569	24	32
	<i>Fusarium roseum</i> ATCC 14717	48	45
	<i>Mortierella isabellina</i> NRRL 1757	48	15
	<i>Fusarium roseum</i> ATCC 14717	48	12
	<i>Cunninghamella bainieri</i> ATCC 9244	72	10
	<i>Cunninghamella bainieri</i> ATCC 9244	72	6
	<i>Absidia corymbifera</i> LCP63.1800	168	10
	<i>Fusarium roseum</i> ATCC 14717	168	8
	<i>Streptomyces rimosus</i> NRRL 2234	96	24

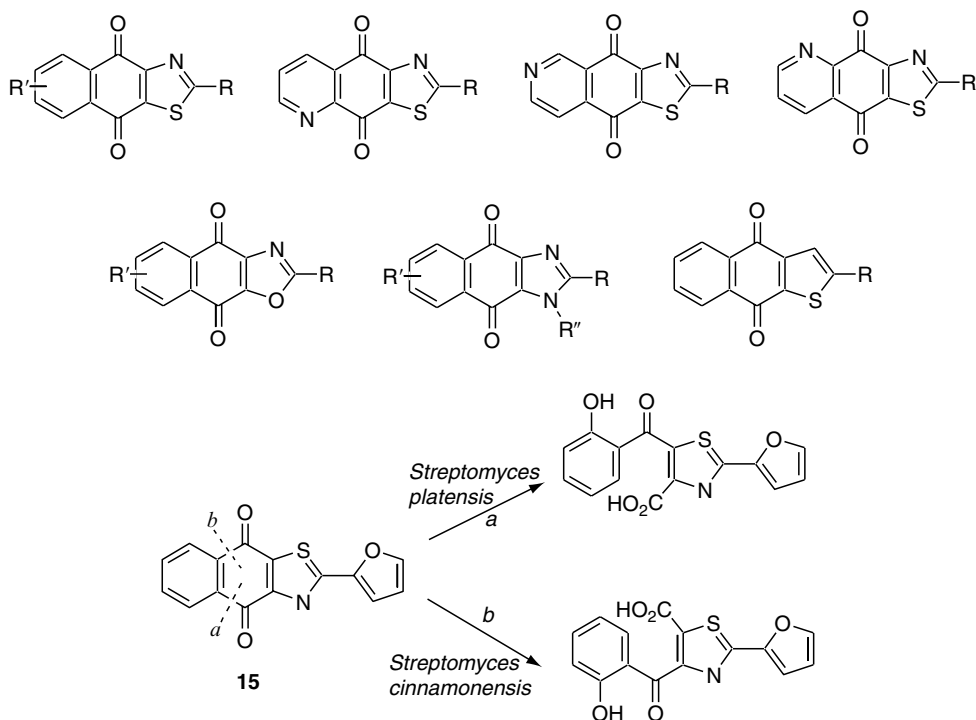


FIGURE 9.15 A library of heterocyclic-substituted naphthoquinones, quinolidione, and isoquinoline-dione analogues, and the oxidative cleavage reactions of INO5042 (**15**) carried out by two *Streptomyces* strains.

detected. In addition, the 1 β -hydroxy derivative exhibited a selective progestomimetic activity comparable to that of the parent compound.

A library of substituted heterocyclic tricyclic 1,4-dioxo-1,4-dihydronaphthalene derivatives and their quinoline and isoquinoline analogs (Figure 9.15) were prepared by Laboratoire Innothera (Arcueil, France) for assaying their pharmacological activities in vein diseases and inflammatory edemas [141]. However, their solubility and biodisponibility were very low and as a possibility to increase their hydrophilicity, biotransformation methods were considered. Surprisingly, a biotransformation study with microorganisms, using naphthothiazole derivative INO5042 (**15**) as a model compound, indicated that several *Streptomyces* strains were able to oxidatively cleave this compound in a dioxygenase-like mechanism [142,143]. Depending on the strain used, two isomeric trifunctional derivatives that might present interesting pharmacological properties were produced in good yields (Figure 9.15). In an approach related to parallel and combinatorial syntheses, the whole library was submitted to biotransformation by the two selected strains, in miniaturized assays, allowing the production, in most cases, of two sublibraries of isomeric oxidized derivatives [144] that thus became available for pharmacological studies.

9.6 CONCLUSION

From the examples, it is clear that microbial transformations (particularly, oxidative transformations related to detoxication mechanisms) may constitute a choice method for the preparation

of new derivatives of complex molecules, without any need for protection or deprotection techniques. Making use of the high activity and versatility of microorganism-catalyzed reactions, in a pseudo-combinatorial approach, a number of novel compounds with potentially new or modified pharmacological or pharmacokinetic activities can be obtained, which becomes available for biological assays. The miniaturization and automation of the microorganism screening, using microwell plates for high throughput culture, incubation, and product recovery and analysis [9,145–147], will certainly alleviate the corresponding labor that represented the main bottleneck of this method. In future, the development of genetic recombinant techniques exploiting the expression of metagenome capabilities [32], associated with combinatorial biosynthesis methods will probably be used for the discovery and preparation of new chemical entities.

REFERENCES

1. Rich, J.O., Michels, P.C., and Khmelnitsky, Y.L., Combinatorial biocatalysis, *Curr. Opin. Chem. Biol.*, 6, 161–167, 2002.
2. Alreuter, D.H. and Clatk, D.S., Combinatorial biocatalysis: taking the lead from nature, *Curr. Opin. Biotechnol.*, 10, 130–136, 1999.
3. Müller, M., Chemical diversity through biotransformations, *Curr. Opin. Biotechnol.*, 15, 591–598, 2004.
4. Michels, P.C., Khmelnitsky, Y.L., Dordick, J.S., et al., Combinatorial biocatalysis: a natural approach to drug discovery, *TIBTECH*, 16, 210–215, 1998.
5. Mozahae, V.V., Budde, C.L., Rich, J.O., et al., Regioselective enzymatic acylation as a tool for producing solution-phase combinatorial libraries, *Tetrahedron*, 54, 3971–3982, 1998.
6. Krstenansky, J.L. and Khmelnitsky, Y.L., Biocatalytic combinatorial synthesis, *Bioorg. Med. Chem.*, 7, 2157–2162, 1999.
7. Tricand de la Goutte, J., Khan, J.A., and Vulfson, E.N., Identification of novel polyphenol oxidase inhibitors by enzymatic one-pot synthesis and deconvolution of combinatorial libraries, *Biotechnol. Bioeng.*, 75, 93–99, 2001.
8. Gebhardt, S., Bihler, S., Shubert-Zsilavec, M., et al., Biocatalytic generation of molecular diversity: modification of ginsenoside Rb1 by β -1,4-galactosyltransferase and *Candida antartica* lipase, Part 4, *Helv. Chem. Acta*, 85, 1943–1959, 2002.
9. Chartrain, M., Greasham, R., Moore, J., et al., Asymmetric bioreduction: application to the synthesis of pharmaceuticals, *J. Mol. Catal., B Enzym.*, 11, 503–512, 2001.
10. Secundo, F., Carrea, G., De Amici, M., et al., A combinatorial biocatalysis approach to an array of cholic acid derivatives, *Biotechnol. Bioeng.*, 81, 391–396, 2003.
11. Lins, R.J., Flitsch, S., Turner, N.J., et al., Generation of a dynamic combinatorial library using sialic acid aldolase and *in situ* screening against wheat germ agglutinin, *Tetrahedron*, 60, 771–780, 2004.
12. Azerad, R., Microbial models for drug metabolism, in *Adv. Biochem. Eng./Biotechnol. (Biotransformations)*, vol. 63, Faber, K. and Scheper, T., Eds., Springer, Heidelberg, 1999, pp. 169–218.
13. Azerad, R., Microbial hydroxylation of terpenoid compounds, in *Stereoselective Biocatalysis*, Patel, R., Ed., Marcel Dekker, New York, 2000, pp. 153–180.
14. Azerad, R., Oxidations using a biocatalyst. Hydroxylation at a saturated carbon, in *Asymmetric Oxidation Reactions: A Practical Approach in Chemistry*, Katsuki, T., Ed., Oxford University Press, New York, 2001, pp. 181–200.
15. Li, Z., Van Beilen, J.B., Duetz, W.A., et al., Oxidative biotransformations using oxygenases, *Curr. Opin. Chem. Biol.*, 6, 136–144, 2002.
16. Urlacher, V., Lutz-Wahl, S., and Schmid, R.D., Microbial P450 enzymes in biotechnology, *Appl. Microbiol. Biotechnol.*, 64, 317–325, 2004.
17. Fura, A., Shu, Y.-Z., Zhu, M., et al., Discovering drugs through biological transformation: role of pharmacologically active metabolites in drug discovery, *J. Med. Chem.*, 47, 4339–4351, 2004.

18. Lacroix, I., Biton, J., and Azerad, R., Microbial models of mammalian metabolism. Microbial transformations of Trimegestone (RU 27987), a 3-keto- $\Delta^{4,9(10)}$ -norsteroid drug, *Bioorg. Med. Chem.*, 7, 2329–2341, 1999.
19. Mahato, S.B., Banerjee, S., and Podder, S., Steroid transformations by microorganisms. III, *Phytochemistry*, 28, 7–40, 1989.
20. Fernandes, P., Cruz, A., Angelova, B., et al., Microbial conversion of steroid compounds: recent developments, *Enzyme Microb. Technol.*, 32, 688–705, 2003.
21. Lamare, V. and Furstoss, R., Bioconversion of sesquiterpenes, *Tetrahedron*, 46, 4109–4132, 1990.
22. Abraham, W.R. and Spassov, G., Biotransformations of alkaloids: a challenge, *Heterocycles*, 56, 711–741, 2002.
23. Vail, R.B., Homann, M.J., Hanna, I., et al., Preparative synthesis of drug metabolites using human cytochrome P450s 3A4, 2C9 and 1A2 with NADPH-P450 reductase expressed in *E. coli*, *J. Ind. Microbiol. Biotechnol.*, 32, 67–74, 2005.
24. Guengerich, F.P., Cytochrome P450 enzymes in the generation of commercial products, *Drug Discovery*, 1, 359–366, 2002.
25. Rushmore, T.H., Reider, P.J., Slaughter, D., et al., Bioreactor systems in drug metabolism: synthesis of cytochrome P450-generated metabolites, *Metabol. Eng.*, 2, 115–125, 2000.
26. Hudlicky, T., Gonzales, D., and Gibson, D.T., Enzymatic dihydroxylation of aromatics in enantioselective synthesis: expanding asymmetric methodology, *Aldrichim. Acta.*, 32, 35–62, 1999.
27. Boyd, D.R. and Sheldrake, G.N., The dioxygenase-catalysed formation of vicinal *cis*-diols, *Nat. Prod. Rep.*, 15, 309–324, 1998.
28. Hutchinson, C.R., Combinatorial biosynthesis for new drug discovery, *Curr. Opin. Microbiol.*, 1, 319–329, 1998.
29. Khosla, C. and Zawada, R.J.X., Generation of polyketide libraries via combinatorial biosynthesis, *TIBTECH*, 14, 335–341, 1996.
30. Rodríguez, E. and McDaniel, R., Combinatorial biosynthesis of antimicrobials and other natural products, *Curr. Opin. Microbiol.*, 4, 526–534, 2001.
31. Weist, S. and Süßmuth, R.D., Mutational biosynthesis—a tool for the generation of structural diversity in the biosynthesis of antibiotics, *Appl. Microbiol. Biotechnol.*, 68, 141–150, 2005.
32. Courtois, S., Cappellano, C.M., Ball, M., et al., Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products, *Appl. Environ. Microbiol.*, 69, 49–55, 2003.
33. Hopwood, D.A., Genetic contributions to understanding polyketide synthases, *Chem. Rev.*, 97, 2465–2497, 1997.
34. Rawlings, B.J., Type I polyketide biosynthesis in bacteria (Part A-erythromycin biosynthesis), *Nat. Prod. Rep.*, 18, 190–227, 2001.
35. Rawlings, B.J., Type I polyketide biosynthesis in bacteria (Part B), *Nat. Prod. Rep.*, 18, 231–281, 2001.
36. Walsh, C.T., Polyketide and nonribosomal peptide antibiotics: modularity and versatility, *Science*, 303, 1805–1810, 2004.
37. Cane, D.E. and Walsh, C.T., The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases, *Chem. Biol.*, 6, R319–R325, 1999.
38. Sieber, S.A. and Marahiel, M.A., Learning from Nature's drug factories: nonribosomal synthesis of macrocyclic peptides, *J. Bacteriol.*, 185, 7036–7043, 2003.
39. Pohl, N.L., Non-natural substrates for polyketide synthases and their associated modifying enzymes, *Curr. Opin. Chem. Biol.*, 6, 773–778, 2002.
40. Pfeifer, B.A. and Khosla, C., Biosynthesis of polyketides in heterologous hosts, *Microbiol. Mol. Biol. Rev.*, 65, 106–118, 2001.
41. Pfeifer, B.A., Admiraal, S.J., Gramajo, H., et al., Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*, *Science*, 291, 1790–1792, 2001.
42. Pfeifer, B., Hu, Z., Licari, P., et al., Process and metabolic strategies for improved production of *Escherichia coli*-derived 6-deoxyerythronolide B, *Appl. Environ. Microbiol.*, 68, 3287–3292, 2002.

43. Harborne, J.B. and Baxter, H., *Handbook of Natural Flavonoids*, vols. 1–2, Wiley, Chichester, 1999.
44. Gao, C., Mayon, P., Macmanus, D.A., et al., Novel enzymatic approach to the synthesis of flavonoid glycosides and their esters, *Biotechnol. Bioeng.*, 71, 235–243, 2001.
45. Danieli, B. and Bertario, A., Chemo-enzymatic synthesis of 6'-O-(3-arylprop-2-enoyl) derivatives of the flavonol glucoside isoquercitrin, *Helv. Chim. Acta*, 76, 2981–2991, 1993.
46. Ardhaoui, M., Falcimagne, A., Engasser, J.M., et al., Enzymatic synthesis of new aromatic and aliphatic esters of flavonoids using *Candida antarctica* lipase as biocatalyst, *Biocatal. Biotransformation.*, 22, 253–259, 2004.
47. Riva, S., Enzymatic modification of the sugar moieties of natural glycosides, *J. Mol. Catal., B Enzym.*, 19–20, 43–54, 2002.
48. Sang, S., Lambert, J.D., Tian, S., et al., Enzymatic synthesis of tea theaflavin derivatives and their anti-inflammatory and cytotoxic activities, *Bioorg. Med. Chem.*, 12, 459–467, 2004.
49. Ibrahim, A.R. and Abul-Hajj, Y.J., Microbiological transformations of (+/–) flavanone and (+/–) isoflavanone, *J. Nat. Prod.*, 53, 644–656, 1990.
50. Ibrahim, A.R. and Habul-Hajj, Y.J., Microbiological transformations of flavone and isoflavone, *Xenobiotica*, 20, 363–373, 1990.
51. Abulhajj, Y.J., Ghaffari, M.A., and Mehrotra, S., Importance of oxygen functions in the biological hydroxylation of flavonoids by *Absidia blackesleeana*, *Xenobiotica*, 21, 1171–1177, 1991.
52. Miyazawa, M., Ando, H., Okuno, Y., et al., Biotransformation of isoflavones by *Aspergillus niger*, as biocatalyst, *J. Mol. Catal., B Enzym.*, 27, 91–95, 2004.
53. Ibrahim, A.R. and Abulhajj, Y.J., Microbiological transformation of chromone, chromanone, and ring A hydroxyflavones, *J. Nat. Prod.*, 53, 1471–1478, 1990.
54. Kostrzewa-Suslow, E., Dmochowka-Gładysz, J., Bialonska, A., et al., Microbial transformations of flavanone and 6-hydroxyflavanone by *Aspergillus niger* cultures, 7th International Symposium on Biocatalysis and Biotransformations (Biotrans' 2005), Delft, The Netherlands, July 3–8, 2005, 190.
55. Ibrahim, A.R., Galal, A.M., Ahmed, M.S., et al., O-demethylation and sulfation of 7-methoxylated flavanones by *Cunninghamella elegans*, *Chem. Pharm. Bull.*, 51, 203–206, 2003.
56. Farooq, A. and Tahara, S., Fungal metabolism of flavonoids and phytoalexins, *Curr. Top. Phytochem.*, 2, 1–33, 1999.
57. Okuno, Y. and Miyazawa, M., Biotransformation of nobiletin by *Aspergillus niger* and the antimutagenic activity of a metabolite, 4'-hydroxy-5,6,7,8,3'-pentamethoxyflavone, *J. Nat. Prod.*, 67, 1876–1878, 2004.
58. Hosny, M., Dhar, K., and Rosazza, J.P.N., Hydroxylations and methylations of quercetin, fisetin, and catechin by *Streptomyces griseus*, *J. Nat. Prod.*, 64, 462–465, 2001.
59. Hosny, M. and Rosazza, J.P.N., Microbial hydroxylations and methylations of genistein by *Streptomyces griseus*, *J. Nat. Prod.*, 62, 1609–1612, 1999.
60. Ibrahim, A.R. and Abulhajj, Y.J., Aromatic hydroxylation and sulfation of 5-hydroxyflavone by *Streptomyces fulvissimus*, *Appl. Environ. Microbiol.*, 55, 3140–3142, 1989.
61. Ibrahim, A.R., Galal, A.M., Mossa, J.S., et al., Glucose-conjugation of the flavones of *Psiadia arabica* by *Cunninghamella elegans*, *Phytochemistry*, 46, 1193–1195, 1997.
62. Ibrahim, A.R., Sulfation of naringenin by *Cunninghamella elegans*, *Phytochemistry* 53, 209–212, 2000.
63. Bayer, T., Colnot, T., and Dekant, W., Disposition and biotransformation of the estrogenic isoflavone daidzein in rats, *Toxicol. Sci.*, 62, 205–211, 2001.
64. Nikolic, D., Li, Y., Chadwick, L.R., et al., Metabolism of 8-prenylnaringenin, a potent phytoestrogen from hops (*Humulus lupulus*) by human liver microsomes, *Drug Metab. Dispos.*, 32, 272–279, 2004.
65. Breinholt, V.M., Rasmussen, S.E., Brosen, K., et al., *In vitro* metabolism of genistein and tangeretin by human and murine cytochrome P450s, *Pharmacol. Toxicol.*, 93, 14–22, 2003.
66. Manach, C. and Donovan, J.L., Pharmacokinetics and metabolism of dietary flavonoids in humans, *Free Radic. Res.*, 38, 771–785, 2004.

67. Nikolic, D. and van Breemen, R.B., New metabolic pathways for flavanones catalyzed by rat liver microsomes, *Drug Metab. Dispos.*, 32, 387–397, 2004.
68. Hur, H.G., Lay, J.O., Beger, R.D., et al., Isolation of human intestinal bacteria metabolizing the natural isoflavone glycosides daidzin and genistin, *Arch. Microbiol.*, 174, 422–428, 2000.
69. Wang, X.-L., Hur, H.-G., Lee, J.H., et al., Enantioselective synthesis of *S*-equol from dihydrodaidzein by a newly isolated anaerobic human intestinal bacterium, *Appl. Environ. Microbiol.*, 71, 214–219, 2005.
70. Klus, K. and Barz, W., Formation of polyhydroxylated isoflavones from the isoflavones genistein and biochanin A by bacteria isolated from tempe, *Phytochemistry*, 47, 1045–1048, 1998.
71. Tahara, S., Saitoh, F., and Mizutani, J., 7-*O*-Methyl-luteone metabolism in *Botrytis cinerea*—identification of the epoxy-intermediate and absolute configuration of the pyrano-isoflavone metabolite, *Z. Naturforsch. C*, 48, 16–21, 1993.
72. Tanaka, M., Mizutani, J., and Tahara, S., Cyclization of a prenylated isoflavone via enzymatic epoxidation, *Biosci. Biotechnol. Biochem.*, 60, 171–172, 1996.
73. Tahara, S., Tanaka, M., and Barz, W., Fungal metabolism of prenylated flavonoids, *Phytochemistry*, 44, 1031–1036, 1997.
74. Sanchez-Gonzalez, M. and Rosazza, J.P.N., Microbial transformations of chalcones: hydroxylation, *O*-demethylation, and cyclization to flavanones, *J. Nat. Prod.*, 67, 553–558, 2004.
75. Kim, S.-Y., Jung, J., Lim, Y., et al., Cis 2',3'-dihydrodiol production on flavone B-ring by biphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 expressed in *E. coli*, *Antonie Van Leeuwenhoek*, 84, 261–268, 2003.
76. Chun, H.-K., Ohnishi, Y., Shindo, K., et al., Biotransformation of flavone and flavanone by *Streptomyces lividans* cells carrying shuffled biphenyl dioxygenase genes, *J. Mol. Catal., B Enzym.*, 21, 113–121, 2003.
77. Shindo, K., Kagiya, Y., Nakamura, R., et al., Enzymatic synthesis of novel antioxidant flavonoids by *Escherichia coli* cells expressing modified metabolic genes involved in biphenyl catabolism, *J. Mol. Catal., B Enzym.*, 23, 9–16, 2003.
78. Seeger, M., Gonzalez, M., Camara, B., et al., Biotransformation of natural and synthetic isoflavonoids by two recombinant microbial enzymes, *Appl. Environ. Microbiol.*, 69, 5045–5050, 2003.
79. Willis, M.G., Giovanni, M., Prata, R.T.N., et al., Bio-fermentation of modified flavonoids: an example of *in vivo* diversification of secondary metabolites, *Phytochemistry*, 65, 31–41, 2004.
80. Tanaka, H., Kuroda, A., Marusawa, A., et al., Structure of FK506: a novel immunosuppressant isolated from *Streptomyces*, *J. Am. Chem. Soc.*, 109, 5031–5033, 1987.
81. Kino, T., Hatanaka, H., Hashimoto, M., et al., FK506, a novel immunosuppressant isolated from a *Streptomyces*, *J. Antibiot.*, 40, 1249–1255, 1987.
82. Hatanaka, H., Kino, T., Miyata, S., et al., FR-900520 and FR-920523, novel immunosuppressants isolated from a *Streptomyces*. I. Taxonomy of the producing strain, *J. Antibiot.*, 41, 1592–1601, 1988.
83. Morisaki, M. and Arai, T., Identity of immunosuppressant FR-900520 with ascomycin, *J. Antibiot.*, 45, 126–128, 1992.
84. Chen, T.S., Li, X., Petuch, B., et al., Structural modification of the immunosuppressants FK-506 and ascomycin using a biological approach., *Chimia*, 53, 596–600, 1999.
85. Inamine, E.S., Wicker, L.S., Chen, S.S.T., et al., Immunosuppressant agent, Eur. Patent 349,061, 1990; *Chem. Abstr.*, 113, 76606z, 1990.
86. Chen, T.S., Arison, B.H., Wicker, L.S., et al., Microbial transformation of immunosuppressive compounds. 1. Desmethylation of FK-506 and immunomycin (FR-900520) by *Actinoplanes* sp. ATCC-53771, *J. Antibiot.*, 45, 118–123, 1992.
87. Chen, T.S., Arison, B.H., Wicker, L.S., et al., Microbial transformation of immunosuppressive compounds. 2. Specific desmethylation of 13-methoxy group of FK-506 and FR-900520 by *Actinomycete* sp. ATCC-53828, *J. Antibiot.*, 45, 577–580, 1992.
88. Shafiee, A., Arison, B.H., Chen, S.S.T., et al., Microbial transformation product having immunosuppressive activity, U.S. Patent 5,352,783, 1994; *Chem. Abst.*, 122, 8154, 1995.

89. Shafiee, A., Chen, T., and Cameron, P., Microbial demethylation of immunosuppressant FK-506: isolation of 31-O-FK-506-specific demethylase showing cytochrome P-450 characteristics from *Streptomyces rimosus* MA187, *Appl. Environ. Microbiol.*, 61, 3544–3548, 1995.
90. Shafiee, A., Chen, T., Arison, B.H., et al., Enzymatic synthesis and immunosuppressive activity of novel desmethylated immunomycins (ascomycins), *J. Antibiot.*, 46, 1397–1405, 1993.
91. Arison, B.H., Inamine, E., Chen, S.S.T., et al., A novel immunosuppressant prepared by microbial transformation of L-683,590, Eur. Patent 378,321, 1990; *Chem. Abst.*, 117, 46696, 1992.
92. Arison, B.H., Inamine, E., Chen, S.S.T., et al., A novel immunosuppressant L-68376 prepared by microbial transformation of L-683,590, Eur. Patent 378,320, 1990; *Chem. Abst.*, 114, 99961, 1991.
93. Arison, B.H., Inamine, E., and Chen, S.S.T., A novel immunosuppressant prepared by microbial transformation of L-679,934, Eur. Patent 378,317, 1990; *Chem. Abstr.* 114, 99960v, 1991.
94. Haag, M., Baumann, K., Billich, A., et al., Bioconversion of ascomycin and its 6-alkoxyderivatives, *J. Mol. Cat., B Enzym.*, 5, 389–394, 1998.
95. Christians, U., Radeke, H.H., Kownatzki, R., et al., Isolation of an immunosuppressive metabolite of FK506 generated by human microsome preparations, *Clin. Biochem.*, 24, 271–275, 1991.
96. Iwasaki, K., Shiraga, T., Nagase, K., et al., Isolation, identification and biological activities of oxidative metabolites of FK506, a potent immunosuppressive macrolide lactone, *Drug Metab. Disp.*, 21, 971–977, 1993.
97. Motamedi, H., Shafiee, A., Cai, S.J., et al., Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK506 and FK520., *J. Bacteriol.*, 178, 5243–5248, 1996.
98. Shafiee, A., Motamedi, H., and Chen, T.S., Enzymology of FK506 biosynthesis: purification and characterization of 31-desmethyl FK506-O-methyltransferase from *Streptomyces* sp. MA6858, *Eur. J. Biochem.*, 225, 764–769, 1994.
99. Chen, T.S., Petuch, B., White, R., et al., Microbial transformation of immunosuppressive compounds. IV. Hydroxylation and hemiketal formation of ascomycin (immunomycin) by *Streptomyces* sp. MA6970 (ATCC no. 55281), *J. Antibiot.*, 47, 1557–1559, 1994.
100. Petuch, B.R., Arison, B.H., Hsu, A., et al., Microbial transformation of immunosuppressive compounds. III. Glucosylation of immunomycin (FR900520) and FK506 by *Bacillus subtilis*, *J. Ind. Microbiol.*, 13, 131–135, 1994.
101. Motamedi, H., Cai, S.J., Shafiee, A., et al., Structural organization of a multifunctional polyketide synthetase involved in the biosynthesis of the macrolide immunosuppressant FK506, *Eur. J. Biochem.*, 244, 74–80, 1997.
102. Motamedi, H. and Shafiee, A., The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506, *Eur. J. Biochem.*, 256, 528–534, 1998.
103. Shafiee, A., Motamedi, H., Dumont, F.J., et al., Chemical and biological characterization of two FK506 analogs produced by targeted gene disruption in *Streptomyces* sp. MA6548, *J. Antibiot.*, 50, 418–423, 1997.
104. Wu, K., Chung, L., Revill, W.P., et al., The FK520 gene cluster of *Streptomyces hygroscopicus* var. *ascomycetinus* (ATCC 14891) contains genes for biosynthesis of unusual polyketide extender units, *Gene*, 251, 81–90, 2000.
105. Revill, W.P., Voda, J., Reeves, C.D., et al., Genetically engineered analogs of ascomycin for nerve regeneration, *J. Pharmacol. Experiment. Therapeut.*, 302, 1278–1285, 2002.
106. Nakagawa, K., Sato, K., Okazaki, T., et al., Microbial conversion of milbemycins—13 β , 29-dihydroxylation of milbemycins by soil isolate *Streptomyces cavourensis*, *J. Antibiot.*, 44, 803–805, 1991.
107. Nakagawa, K., Sato, K., Tsukamoto, Y., et al., Microbial conversion of milbemycins—microbial conversion of milbemycins A4 and A3 by *Streptomyces libani*, *J. Antibiot.*, 47, 502–506, 1994.
108. Nakagawa, K., Sato, K., Tsukamoto, Y., et al., Microbial conversion of milbemycins: 28-hydroxylation of milbemycins by *Amycolata autotrophica*, *J. Antibiot.*, 46, 518–519, 1993.
109. Nakagawa, K., Miyakoshi, S., Torikata, A., et al., Microbial conversion of milbemycins—hydroxylation of milbemycin-A4 and related compounds by *Cunninghamella echinulata* ATCC 9244, *J. Antibiot.*, 44, 232–240, 1991.

110. Nakagawa, K., Sato, K., Tsukamoto, Y., et al., Microbial conversion of milbemycins: 29-hydroxylation of milbemycins by genus *Syncephalastrum*, *J. Antibiot.*, 45, 802–805, 1992.
111. Nakagawa, K., Torikata, A., Sato, K., et al., Microbial conversion of milbemycins: 30-oxidation of milbemycin-A4 and related compounds by *Amycolata autotrophica* and *Amycolatopsis mediterranei*, *J. Antibiot.*, 43, 1321–1328, 1990.
112. Nakagawa, K., Sato, K., Tsukamoto, Y., et al., Microbial conversion of milbemycins: oxidation of milbemycin A4 and related compounds at the C-25 ethyl group by *Circinella umbellata* and *Absidia cylindrospora*, *J. Antibiot.*, 48, 831–837, 1995.
113. Ramos Tombo, G.M., Ghisalpa, O., Schär, H.-P., et al., Diastereoselective microbial hydroxylation of milbemycin derivatives, *Agric. Biol. Chem.*, 53, 1531–1535, 1989.
114. Schulman, M., Doherty, P., and Arison, B., Microbial conversion of avermectins by *Saccharopolyspora erythraea*—glycosylation at C-4' and C-4'', *Antimicrob. Agents Chemother.*, 37, 1737–1741, 1993.
115. Wei, L., Wei, G., Zhang, H., et al., Synthesis of new, potent avermectin-like insecticidal agents, *Carbohydr. Res.*, 340, 1583–1590, 2005.
116. Tsukamoto, Y., Nakagawa, K., Kajino, H., et al., Synthesis of novel 25-substituted milbemycin A4 derivatives and their acaricidal activity against *Tetranychus urticae*, *Biosci. Biotechnol. Biochem.*, 61, 1650–1657, 1997.
117. Schulman, M., Doherty, P., Zink, D., et al., Microbial conversion of avermectins by *Saccharopolyspora erythraea*: hydroxylation at C-27, *J. Antibiot.*, 47, 372–375, 1994.
118. Chartrain, M., White, R., Goegelman, R., et al., Bioconversion of avermectin into 27-OH avermectin, *J. Ind. Microbiol.*, 6, 279–284, 1990.
119. Arison, B.H., Doherty, P.J., and Schulman, M.D., Bioconversion products of 27-hydroxy avermectin, Brit. Patent 2,281,911, 1995; *Chem. Abstr.*, 123, 54274u, 1995.
120. Schulman, M., Doherty, P., Zink, D., et al., Microbial conversion of avermectins by *Saccharopolyspora erythraea*: hydroxylation at C28, *J. Antibiot.*, 46, 1016–1019, 1993.
121. Arison, B.H., Doherty, P.J., and Schulman, M.D., Fermentation of 28-hydroxy ivermectin aglycone with *Saccharopolyspora*, U.S. Patent 5,124,258, 1992; *Chem. Abstr.*, 117, 169566j, 1992.
122. McNeil, D.J., Occi, J.L., Gewain, K.M., et al., Correlation of the avermectin polyketide synthase genes to the avermectin structure. Implications for designing novel avermectins, *Ann. N.Y. Acad. Sci.*, 721, 123–132, 1994.
123. Ikeda, H. and Omura, S., Avermectin biosynthesis, *Chem. Rev.*, 97, 2591–2609, 1997.
124. Ikeda, H., Tonomiya, T., and Omura, S., Organization of biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*: analysis of enzymatic domains in four polyketide synthases, *J. Ind. Microbiol. Biotechnol.*, 27, 170–176, 2001.
125. Ikeda, H., Tonomiya, T., Usami, M., et al., Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*, *Proc. Natl. Acad. Sci. USA*, 96, 9509–9514, 1999.
126. Omura, S., Ikeda, H., Ishikawa, J., et al., Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites, *Proc. Natl. Acad. Sci. USA*, 98, 12215–12220, 2001.
127. Ikeda, H., Ishikawa, J., Hanamoto, A., et al., Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*, *Nat. Biotechnol.*, 21, 526–531, 2003.
128. Yoon, Y.J., Kim, E.-S., Hwang, Y.-S., et al., Avermectin: biochemical and molecular basis for its biosynthesis and regulation, *Appl. Microbiol. Biotechnol.*, 63, 626–634, 2004.
129. Chen, T.S., Inamine, E.S., Hensens, O.D., et al., Directed biosynthesis of avermectins, *Arch. Biochem. Biophys.*, 269, 544–547, 1989.
130. Hafner, E.W., Holley, B.W., Holdom, K.S., et al., Branched-chain fatty acid requirement for avermectin production by a mutant of *Streptomyces avermitilis* lacking branched-chain 2-oxo acid dehydrogenase activity, *J. Antibiot.*, 44, 349–356, 1991.
131. Dutton, C.J., Gibson, S.P., Goudie, A.C., et al., Novel avermectins produced by mutational biosynthesis, *J. Antibiot.*, 44, 357–365, 1991.
132. Denoya, C.D., Fedechko, R., Hafner, E.W., et al., A second branched-chain α -keto acid dehydrogenase gene cluster (*bkdFGH*) from *Streptomyces avermitilis*: its relationship to avermectin

- biosynthesis and the construction of a *bkdF* mutant suitable for the production of novel antiparasitic avermectins, *J. Bacteriol.*, 177, 3504–3511, 1995.
133. Cropp, T.A., Wilson, D.J., and Reynolds, K.A., Identification of a cyclohexycarbonyl CoA biosynthetic gene cluster and application in the production of doramectin, *Nat. Biotechnol.*, 18, 980–983, 2000.
 134. Stutzman-Engwall, K., Conlon, S., Fedechko, R., et al., Semi-synthetic DNA shuffling of *aveC* leads to improved industrial scale production of doramectin by *Streptomyces avermitilis*, *Metabol. Eng.*, 7, 27–37, 2005.
 135. Ikeda, H., Wang, L.-R., Ohta, T., et al., Cloning of the gene encoding avermectin B 5-*O*-methyltransferase in avermectin-producing *Streptomyces avermitilis*, *Gene*, 206, 175–180, 1998.
 136. Tsukamoto, Y., Sato, K., Mio, S., et al., Synthesis of 5-keto-5-oxime derivatives of milbemycins and their activities against microfilariae, *Agric. Biol. Chem.*, 55, 2615–2621, 1991.
 137. Gaisser, S., Kellenberger, L., Kaja, A.L., et al., Direct production of ivermectin-like drugs after domain exchange in the avermectin polyketide synthase of *Streptomyces avermitilis* ATCC 31272, *Org. Biomol. Chem.*, 1, 2840–2847, 2003.
 138. Yong, J.H. and Byeon, W.H., Alternative production of avermectin components in *Streptomyces avermitilis* by gene replacement, *J. Microbiol.*, 43, 277–284, 2005.
 139. Hong, Y.-S., Hwang, B.Y., Kim, H.S., et al., Biotransformation of Milbemycin D to Milbemycin G by *Streptomyces lividans* conferring avermectin *O*-methyltransferase activity, *Biotechnol. Lett.*, 20, 991–995, 1998.
 140. Biton, J., Azerad, R., Marchandau, J.P., et al., Process for the preparation of new 1- or 6-hydroxylated steroids and their use as medicaments, Eur. Patent 808,845, 1997; *Chem. Abstr.*, 128, 34924, 1997.
 141. Bouterin-Falson, O., Desquand-Billiald, S., Favrou, A., et al., Preparation of heterocyclic tricyclic 1,4-dihydro-1,4-dioxonaphthalene vasoconstrictors, PCT Appl. WO 9721684, 1997; *Chem. Abstr.*, 127, 121–716, 1997.
 142. Le Texier, L., Roy, S., Fosse, C., et al., A biosynthetic microbial ability applied to the oxidative ring cleavage of non-natural heterocyclic quinones, *Tetrahedron Lett.*, 42, 4135–4137, 2001.
 143. Fosse, C., Le Texier, L., Roy, S., et al., Parameters and mechanistic studies on the oxidative ring cleavage of synthetic heterocyclic naphthoquinones by *Streptomyces* strains, *Appl. Microbiol. Biotechnol.*, 65, 446–456, 2004.
 144. Fosse, C., Le Texier, L., Roy, S., et al., unpublished results.
 145. Duetz, W.A., Minas, W., Kühner, M., et al., Miniaturized microbial growth systems in screening, *Bio World*, 2, 8–10, 2001.
 146. Clerval, R., Kühner, M., Li, Z., et al., The come-back of high throughput screening of wild-type microbial strains through the use of miniaturised growth systems and LC-MS, *Bio World*, 6, 24–26, 2001.
 147. Lye, G.J., Ayazi-Shamlou, P., Baganz, F., et al., Accelerated design of bioconversion processes using automated microscale processing techniques, *TIBTECH*, 21, 29–37, 2003.
 148. Arison, B.H., Inamine, E., Chen, S.S.T., et al., Microbial transformation product of L-679,934, Eur. Patent 378,317, 1990.
 149. Chen, S.S.T., White, R.F., Dezeny, G., et al., New C-31 desmethyl FR-900520 cyclic hemiketal immunosuppressant agent, Eur. Patent 526,934, 1993; *Chem. Abstr.*, 118, 190103, 1993.
 150. Shafiee, A., Kaplan, L., Chen, S.S.T., et al., C-31-methylated FR-900520 cyclic hemiketal immunosuppressant agents obtained by fermentation, U.S. Patent 5,149,701 A, 1992; *Chem. Abstr.*, 118, 32932, 1993.
 151. Shafiee, A., Kaplan, L., Chen, S.S.T., et al., Manufacture of derivatives of FR-520 for use as immunosuppressants with *Streptomyces lavendulae*, Eur. Patent 526,935 A1, 1993; *Chem. Abstr.*, 118, 190104, 1993.

10 Applications of Aromatic Hydrocarbon Dioxygenases

Rebecca E. Parales and Sol M. Resnick

CONTENTS

10.1	Introduction	299
10.2	Distribution and Diversity	300
10.2.1	Distribution and Detection of Aromatic-Ring-Hydroxylating Dioxygenases in Bacteria	300
10.2.2	Types of Reactions Catalyzed	301
10.3	Characteristics of Aromatic-Ring-Hydroxylating Dioxygenases	301
10.3.1	Multicomponent Nature	304
10.3.2	Broad Substrate Range	304
10.3.3	Regioselectivity and Enantioselectivity	305
10.4	Biocatalysis Strategies	309
10.4.1	Whole-Cell Bioprocesses	309
10.4.2	Cofactor Regeneration	310
10.4.3	Reaction and Process Engineering Approaches	310
10.4.4	Biocatalyst Improvement	311
10.5	Applications	312
10.5.1	Enantioselective Synthesis Using Dioxygenase-Derived Synthons	312
10.5.2	Dioxygenase-Catalyzed Reactions for Lead Development and Natural Product Modification	313
10.5.3	Indinavir Production	315
10.5.4	Indigo Production	315
10.5.5	Bioremediation of TCE Contamination	315
10.5.6	Construction of New Biodegradation Pathways	316
10.6	Outlook	316
	Acknowledgments	317
	References	317

10.1 INTRODUCTION

Aromatic hydrocarbon dioxygenases refer specifically to enzymes that catalyze the oxidation of the aromatic ring of aromatic hydrocarbons, compounds containing only carbon and hydrogen. This group of enzymes represents an important subset of a large family of “aromatic-ring-hydroxylating dioxygenases” or “Rieske nonheme iron oxygenases” as they have been variously designated, all with similar mechanism and structure [1,2]. Aromatic-ring-hydroxylating dioxygenases initiate bacterial pathways for the degradation of a wide range of aromatic compounds, including polycyclic aromatic hydrocarbons, nitroaromatic and

chlorinated aromatic compounds, aromatic acids, and heterocyclic aromatic compounds. Toluene dioxygenase (TDO) from *Pseudomonas putida* F1 was the first characterized aromatic hydrocarbon dioxygenase. Identified by Gibson and coworkers, TDO [3,4] was shown to catalyze *cis*-dihydroxylation of benzene and toluene [5–7]. Our understanding of the structure, function, and substrate specificity of aromatic hydrocarbon dioxygenases has come from the studies of TDO and naphthalene dioxygenase (NDO). To date, more than 100 Rieske nonheme iron oxygenases have been identified on the basis of biological activity or nucleotide sequence identity. These multicomponent enzyme systems are cofactor requiring proteins (EC 1.14.12.-) that catalyze the addition of molecular oxygen to the aromatic ring through reductive dihydroxylation. The initial oxidation of the aromatic ring is the most difficult step in the degradation of this class of compounds. This addition of hydroxyl groups to the highly stable aromatic ring activates the molecule for further oxidation and eventual ring cleavage. Aromatic-ring-hydroxylating dioxygenases are unrelated in both structure and function to aromatic-ring-cleavage (or ring-fission) dioxygenases (EC 1.13.11.-) that oxidize and cleave the catecholic intermediates produced during the catabolism of aromatic compounds.

10.2 DISTRIBUTION AND DIVERSITY

Many of the substrates for aromatic-ring-hydroxylating dioxygenases are toxic environmental pollutants. Some, such as certain chlorinated and nitroaromatic compounds, are synthetic, while others are naturally occurring biological or pyrolysis products, or components of petroleum [8]. Bacterial degradation of these chemicals is critical for recycling of carbon on earth as well as removal of toxic pollutants at contaminated sites.

10.2.1 DISTRIBUTION AND DETECTION OF AROMATIC-RING-HYDROXYLATING DIOXYGENASES IN BACTERIA

Most aromatic-ring-hydroxylating dioxygenases have been identified from bacterial isolates capable of growth on specific aromatic compounds. These strains are typically isolated by selective enrichment and subsequent plating on minimal medium containing the aromatic substrate as the sole source of carbon. Depending on their vapor pressures and solubilities, various substrates have been incorporated into the agar media, provided in the vapor phase [9], or sprayed as an insoluble layer onto the plate surface [10]. Bacteria capable of degrading many naturally occurring aromatic compounds can be easily isolated from most soil samples, but other good sources of inocula include samples from sites with a history of exposure to environmental pollutants such as creosote, gasoline, refined petroleum products, or other aromatic pollutants. A wide range of both Gram-positive and Gram-negative bacteria have been found to harbor aromatic compound degradation pathways that are initiated by ring-hydroxylating dioxygenases, although many of the isolates are pseudomonads or related proteobacteria. This isolation bias is due to the rapid doubling times and minimal growth factor requirements exhibited by this group of bacteria that enable them to dominate enrichment cultures. Recently, there have been more efforts to characterize the diversity of organisms capable of degrading aromatic compounds, and a wider range of genera are now known to utilize dioxygenase-mediated degradation pathways.

Several colorimetric indicators are available for detecting dioxygenase activity or activity of downstream enzymes required for aromatic compound degradation. These include the well-known conversion of indole to indigo [11], which is catalyzed by many aromatic hydrocarbon dioxygenases, and the conversion of indole carboxylic acids to indigo by certain aromatic acid dioxygenases [12]. The detection of ring-cleavage products formed from the catecholic intermediates of aromatic compound degradation has been used to identify

coupled activities of aromatic-ring-hydroxylating dioxygenases, *cis*-dihydrodiol dehydrogenases (DDH), and *meta* ring-cleavage dioxygenases [13]. Gene-based discovery approaches for the detection of aromatic hydrocarbon dioxygenases can be employed for screening libraries prepared directly from environmental samples. However, several challenges exist for the application of these techniques to multicomponent enzyme systems. Activity screens require that each gene is present and sufficiently expressed for detection of activity. Homology-based hybridization and PCR amplification typically do not allow the identification of high-genetic diversity, and hybridization of multiple components can be complicated by the “mosaic” organization of the encoding genes [14]. One approach identified novel polycyclic aromatic hydrocarbon degradation genes by screening the genomic DNA from aromatic hydrocarbon degrading isolates for lack of hybridization to standard dioxygenase gene probes [15].

10.2.2 TYPES OF REACTIONS CATALYZED

The primary reaction catalyzed by aromatic-ring-hydroxylating dioxygenases on aromatic substrates is a *cis*-dihydroxylation of the carbon–carbon double bond of adjacent unsubstituted carbon atoms. In most cases, this reaction results in the formation of a stable chiral *cis*-dihydrodiol (Reaction A, Table 10.1). Similarly, oxidation of aromatic acids such as benzoate occurs at the carboxylated carbon and an adjacent unsubstituted carbon, and a chiral *cis*-dihydroxylated cyclohexadiene carboxylic acid is formed (Reaction B, Table 10.1). Dioxygenase-catalyzed dechlorination can occur with chlorinated benzoates, benzenes, and biphenyls as substrates; dioxygenation at a chlorine-substituted carbon results in the elimination of chloride and formation of a catechol (Reactions C and D, Table 10.1) [16–19]. Nitrite, ammonia, or sulfite elimination has been demonstrated with certain enzymes when provided with nitroaromatic, aminoaromatic, or sulfoaromatic substrates (Reactions E–H, Table 10.1) [20–25]. With these substrates, the displacement reactions also result in the formation of catecholic products. Certain members of this large family of multicomponent enzymes catalyze angular dioxygenation of certain multiring substrates [26,27] (Reaction I, Table 10.1). Substrates that are oxidized by angular dioxygenation include carbazole, diphenylethers, dibenzofuran, and dibenzo-*p*-dioxin [28–32]. In addition, a small subset of ring hydroxylating dioxygenases function as monooxygenases with their native substrates. Such enzymes include salicylate 5-hydroxylase [33] and salicylate 1-hydroxylase [34–36] (Reaction P, Table 10.1), 2-oxo-1,2-dihydroquinoline-8-monooxygenase [37] and toluene sulfonate methyl-monooxygenase [38] (Reaction K, Table 10.1), methoxybenzoate monooxygenase [39], vanillate demethylase [40–44], and 5,5'-dehydrodivanillic acid *O*-demethylase [45], the latter three reactions involving an *O*-demethylation (Reaction N, Table 10.1). Three other reaction types have been documented primarily for NDO, TDO, and in some cases, carbazole dioxygenase. These include benzylic hydroxylation [46–54] (Reaction J, Table 10.1), oxygen-dependent desaturation [46,51,52,54,55] (Reaction L, Table 10.1), sulfoxidation [13,49,50,56–58] (Reaction M, Table 10.1), and *N*-dealkylation [59,60] (Reaction O, Table 10.1). Finally, dioxygen-dependent alcohol oxidation (Reaction Q, Table 10.1) has been reported for purified NDO [46].

10.3 CHARACTERISTICS OF AROMATIC-RING-HYDROXYLATING DIOXYGENASES

Aromatic-ring-hydroxylating dioxygenases are capable of oxidizing a wide range of substrates [60,61]. Specific enzymes demonstrate a remarkable diversity both in the number of substrates oxidized and in the types of reactions catalyzed. Our understanding of the substrate

TABLE 10.1
Reactions Catalyzed by Aromatic-Ring-Hydroxylating Dioxygenases

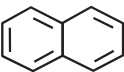
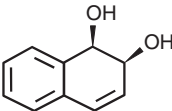
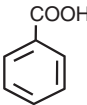
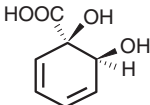
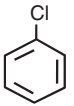
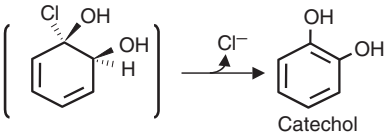
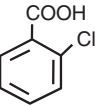
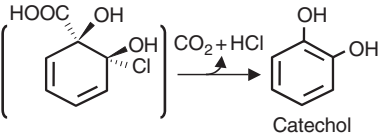
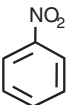
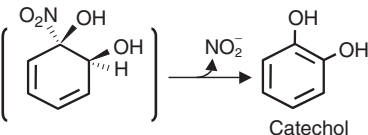
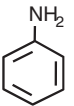
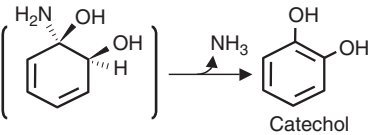
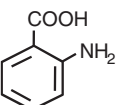
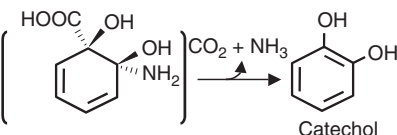
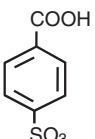
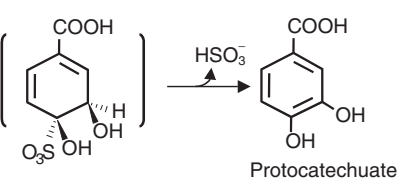
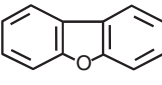
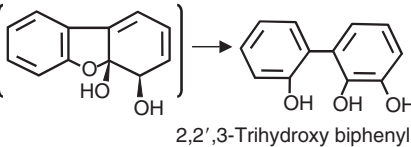
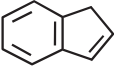
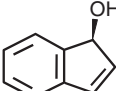
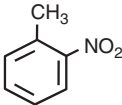
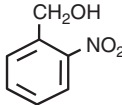
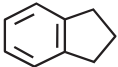
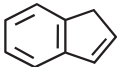
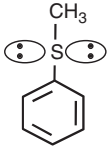
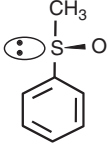
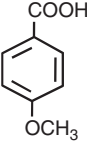
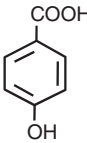
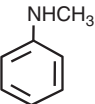
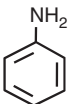
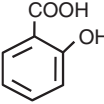
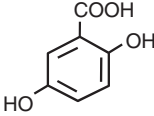
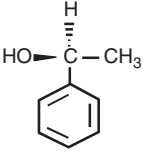
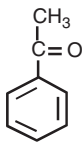
Reaction Type	Substrate	Enzyme	Product
A. <i>cis</i> -Dihydroxylation	 Naphthalene	Naphthalene dioxygenase $\xrightarrow{O_2}$	 Naphthalene <i>cis</i> -1,2-dihydrodiol
B. <i>cis</i> -Dihydroxylation	 Benzoate	Benzoate dioxygenase $\xrightarrow{O_2}$	 Benzoate <i>cis</i> -1,2-dihydrodiol
C. <i>cis</i> -Dihydroxylation and dehalogenation	 Chlorobenzene	Chlorobenzene dioxygenase $\xrightarrow{O_2}$	 Catechol
D. <i>cis</i> -Dihydroxylation, dehalogenation and decarboxylation	 2-Chlorobenzoate	Chlorobenzoate dioxygenase $\xrightarrow{O_2}$	 Catechol
E. <i>cis</i> -Dihydroxylation and nitrite elimination	 Nitrobenzene	Nitrobenzene dioxygenase $\xrightarrow{O_2}$	 Catechol
F. <i>cis</i> -Dihydroxylation and deamination	 Aniline	Aniline dioxygenase $\xrightarrow{O_2}$	 Catechol
G. <i>cis</i> -Dihydroxylation, deamination and decarboxylation	 Anthranilate	Anthranilate dioxygenase $\xrightarrow{O_2}$	 Catechol
H. <i>cis</i> -Dihydroxylation, and desulfonation	 <i>p</i> -Sulfobenzoate	<i>p</i> -Sulfobenzoate dioxygenase $\xrightarrow{O_2}$	 Protocatechuate
I. Angular dihydroxylation	 Dibenzofuran	Dibenzofuran dioxygenase $\xrightarrow{O_2}$	 2,2',3-Trihydroxy biphenyl

TABLE 10.1 (continued)
Reactions Catalyzed by Aromatic-Ring-Hydroxylating Dioxygenases

Reaction Type	Substrate	Enzyme	Product
J. Benzylic hydroxylation	 Indene	$\xrightarrow[\text{O}_2]{\text{Naphthalene dioxygenase}}$	 Indenol
K. Methyl group hydroxylation	 2-Nitrotoluene	$\xrightarrow[\text{O}_2]{\text{Naphthalene dioxygenase}}$	 2-Nitrobenzyl alcohol
L. Oxygen-dependent desaturation	 Indan	$\xrightarrow[\text{O}_2]{\text{Naphthalene dioxygenase}}$	 Indene
M. Sulfoxidation	 Methyl phenyl sulfide	$\xrightarrow[\text{O}_2]{\text{Naphthalene dioxygenase}}$	 Methyl phenylsulfoxide
N. O-Dealkylation	 4-Methoxybenzoate	$\xrightarrow[\text{O}_2]{\text{Naphthalene dioxygenase}}$	 4-Hydroxybenzoate
O. N-Dealkylation	 N-Methylaniline	$\xrightarrow[\text{O}_2]{\text{Naphthalene dioxygenase}}$	 Aniline
P. Net aromatic-ring hydroxylation	 Salicylate	$\xrightarrow[\text{O}_2]{\text{Salicylate 5-hydroxylase}}$	 Gentisate
Q. Dioxygen- dependent alcohol oxidation	 (S)-1-Phenethyl alcohol	$\xrightarrow[\text{O}_2]{\text{Naphthalene dioxygenase}}$	 Acetophenone

Source: Adapted from Parales, R.E. and Resnick, S.M. in *Pseudomonas Volume 4: Molecular Biology of Emerging Issues*, Academic/Plenum, New York, 2006. With permission.

specificities of various aromatic hydrocarbon dioxygenases has been based on the studies with wild-type strains, blocked mutant strains that accumulate the products of dioxygenase-catalyzed reactions, recombinant strains expressing cloned dioxygenase genes, and in some cases with purified enzymes. While over 100 aromatic-ring-hydroxylating dioxygenases have been reported, much of our knowledge on substrate specificity has been obtained through detailed studies with relatively few enzymes that include TDOs, NDOs, biphenyl dioxygenases (BPDOs), chlorobenzene dioxygenase, and carbazole dioxygenase. The range of substrates and selectivity of reactions catalyzed make these aromatic hydrocarbon dioxygenases particularly interesting for a variety of applications.

10.3.1 MULTICOMPONENT NATURE

Rieske nonheme iron oxygenases are two- or three-component enzyme systems. All have either one or two electron transport proteins that transfer electrons from NAD(P)H to the catalytic oxygenase component. Therefore, to set up an *in vitro* biotransformation system, at least two proteins must be purified and recombined in an optimized ratio. In addition, a source of reductant is continuously required. To date, protein components from several aromatic hydrocarbon dioxygenase systems have been purified and studied in detail, including those of the NDO system from *Pseudomonas* sp. NCIB 9816-4 [62–64]. During the reaction catalyzed by NDO, electrons are transferred sequentially from NAD(P)H to the reductase, to the ferredoxin, to the Rieske center of the oxygenase and finally to the iron at the active site (Figure 10.1). Two electrons are necessary to complete the reaction cycle [65]. The 35 kD reductase contains one molecule of FAD and a plant-type iron–sulfur center; it can accept electrons from either NADH or NADPH [63,66]. The Rieske iron–sulfur center containing ferredoxin is a monomer of approximately 11 kD [62,66]. The oxygenase is an $\alpha_3\beta_3$ hexamer [67] in which each α subunit contains a Rieske [2Fe-2S] center and mononuclear Fe^{2+} at the active site. When individual subunits of the oxygenase were purified and reconstituted [68,69], both subunits were found to be essential for activity; similar results were seen with TDO [70] and BPDO [71].

10.3.2 BROAD SUBSTRATE RANGE

The substrate specificities of several aromatic-ring-hydroxylating dioxygenases have been investigated in detail. NDO from *Pseudomonas* sp. NCIB 9816-4 catalyzes the oxidation of more than 75 different substrates [60] by not only *cis*-dihydroxylation (dioxygenation) reactions, but also benzylic monohydroxylation (monooxygenation [53,72]), desaturation [52,54], *O*- and *N*-dealkylation [55,59], and sulfur oxidation (sulfoxidation [58]) reactions (Table 10.1). TDO has the ability to catalyze the same range of reaction types as NDO

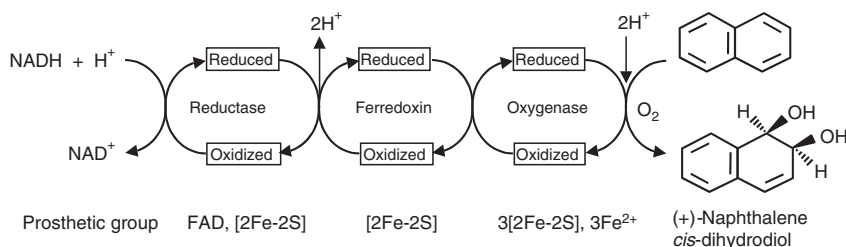


FIGURE 10.1 Reaction catalyzed by the three-component NDO enzyme system from *Pseudomonas* sp. NCIB 9816-4.

(Table 10.1). TDOs from *P. putida* F1 and *P. putida* UV4 have equally impressive substrate ranges, with the ability to catalyze the oxidation of more than 100 substrates, including monocyclic aromatic compounds, polycyclic and heterocyclic aromatic compounds, substituted aromatics, conjugated mono- and polyalkenes, and a variety of halogenated and non-halogenated aliphatic olefins [73–76]. Table 10.2 shows an extensive list of substrates oxidized by aromatic-ring-hydroxylating dioxygenases, the reaction types catalyzed (Table 10.1), and relevant references, which indicate the enzyme(s) known to catalyze the reactions. The specificities of NDO and TDO often overlap, but in general, TDO prefers smaller (1–3–ring) substrates, while NDO prefers larger (2–4–ring) substrates. The BPDO from *Sphingomonas yanoikuyae* strain B1 (isolated for its ability to grow on biphenyl) has been shown to catalyze *cis*-dihydroxylation of a wide range of polycyclic and heterocyclic aromatic compounds including not only naphthalene, phenanthrene, anthracene, acenaphthene, but also high molecular weight polycyclics such as benzo[a]pyrene, benzo[a]anthracene, chrysene and benzo[b]-naphtha[2,1-d]thiophene [77–82]. In fact, *cis*-dihydrodiols from the latter two substrates as well as from acridine and phenazine undergo sequential *cis*-dihydroxylation to yield *bis-cis*-diol metabolites [81,83,84]. The substrate specificity of carbazole dioxygenase from *P. resinovorans* CA10 has also been investigated. This enzyme, which catalyzes angular dioxygenation (Reaction I, Table 10.1) on its native substrate, is capable of both *cis*-dihydroxylation and angular dioxygenation with several aromatic hydrocarbons and heterocycles [56].

10.3.3 REGIOSELECTIVITY AND ENANTIOSELECTIVITY

As a group, aromatic-ring-hydroxylating dioxygenases exhibit relaxed specificity as evidenced by their vast and structurally diverse substrate range. Yet, the substrates that are accepted are generally oxidized with both high regioselectivity and high enantioselectivity. Studies with different dioxygenases and a series of benzocyclic aromatic compounds suggest that the types of reactions catalyzed are determined by the fit of the given molecule within the active site. Examples of this point are illustrated by detailed characterization of oxidation products formed by TDO and NDO from indan, indene and related compounds. TDO oxidizes indan to (1*R*)-indanol and converts indene to *cis*-(1*S*, 2*R*)-indandiol and (1*R*)-indenol [53]. In contrast, NDO oxidizes indan to (1*S*)-indanol and oxidizes indene to *cis*-(1*R*, 2*S*)-indandiol and (1*S*)-indenol [52]. Such studies also illustrate the trends observed in product enantioselectivity for specific reaction types that is often a conserved intrinsic characteristic for a particular dioxygenase.

Some of the trends in enantioselectivity have been well characterized for the *cis*-dihydroxylation, benzylic monohydroxylation and sulfoxidation reactions catalyzed by NDO [60], and other aromatic-ring-hydroxylating dioxygenases. For example, NDO typically catalyzes benzylic hydroxylation and sulfoxidation to yield benzylic alcohols and sulfoxides of (*S*)-configuration [60,76]. In contrast, TDO typically catalyzes benzylic hydroxylation and sulfoxidation to generate products of (*R*)-configuration [57,58,85]. This enantiocomplementarity for TDO and NDO has been documented for benzylic hydroxylation of 2-indanol [86] and chiral indanols [87]. Other organisms have been identified that are capable of generating enantiopure *cis*-diols of opposite chirality to that of previously identified metabolites. For example, a carbazole-utilizing strain oxidized biphenyl, biphenylene, and 9-fluorenone to previously unobserved *cis*-dihydrodiol or angular monohydrodiol enantiomers [88,89].

Recent reports showed that strains expressing TDO and NDO catalyze dihydroxylation of conjugated monoalkene and polyalkenes to yield the corresponding monols and enantiopure cyclic *cis*-diols [76]. Both the dioxygenase and the alkene substrates were important factors in determining the preference for 1,2-dihydroxylation of conjugated alkene or arene groups, and monohydroxylation at benzylic or allylic centers. These factors are illustrated with styrene and a

[illegible]

continued

TABLE 10.2 (contiued)
Reactions Catalyzed by Rieske Nonheme Iron Oxygenases for Specific Substrates

Substrate	Type of Oxidation Reaction ^a																
	Dihydroxylations									Other Oxidations							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
2-Oxo-1,2-dihydroquinoline [37,263]																✓	
Pyrazon [264]	✓																
3-Methyl benzothiophene [49]											✓		✓				
2-Methylbenzo-1,3-thiole [57]													✓				
Carbocyclic, alkyl-aryl ether, thioether, or N-alkyl substrates																	
Indan [51–53]										✓		✓					
Indene [52,144,265]	✓									✓							
1,2-Dihydronaphthalene [54,266]	✓									✓		✓					
Tetralin [267,268]	✓																
Methyl phenyl sulfide [57,58]													✓				
Ethyl phenyl sulfide [57,58]													✓				
Methyl <i>p</i> -tolyl sulfide [58]													✓				
<i>p</i> -Methoxyphenyl methyl sulfide [58]													✓				
Methyl <i>p</i> -nitrophenyl sulfide [58]													✓				
Anisole [55]	✓														✓		
Phenetole [55]	✓											✓			✓		
Carboxydiphenylethers [269]									✓								
<i>N</i> -Methylindole [60]																✓	
<i>N</i> -Methylaniline [59]																✓	
<i>N,N</i> -Dimethylaniline [59]																✓	

^aThe types of reactions are shown in Table 10.1: A, *cis*-Dihydroxylation (C=C); B, *cis*-Dihydroxylation (at and adjacent to a carboxyl bearing carbon); C, *cis*-Dihydroxylation and dehalogenation; D, *cis*-Dihydroxylation, decarboxylation, and dehalogenation; E, *cis*-Dihydroxylation and nitrite elimination; F, *cis*-Dihydroxylation and deamination; G, *cis*-Dihydroxylation, deamination, and decarboxylation; H, Dihydroxylation and desulfonation; I, Angular dihydroxylation; J, Benzylic hydroxylation; K, Methyl group hydroxylation; L, Oxygen-dependent desaturation; M, Sulfoxidation; N, *O*-Dealkylation; O, *N*-Dealkylation; P, Net aromatic-ring monohydroxylation; Q, Dioxygen-dependent alcohol oxidation. This list is not exhaustive and additional information can be found in recent reviews.

Source: Resnick, S. M., Lee, K., and Gibson, D.T., *J. Ind. Microbiol.*, 17, 438–457, 1996; Hudlicky, T., Gonzalez, D., and Gibson, D.T., *Aldrichimica Acta*, 32, 35–62, 1999; Boyd, D.R., and Sheldrake, G.N., *Nat. Prod. Rep.*, 15, 309–324, 1998; Boyd, D.R., Sharma, N.D., and Allen, C.C.R., *Curr. Opin. Biotechnol.*, 12, 564–573, 2001; Parales, R.E. and Resnick, S.M. in *Pseudomonas Volume 4: Molecular Biology of Emerging Issues*, Academic/Plenum, New York, 2006. With permission.

series of *meta*-substituted styrenes that were substrates for TDO-catalyzed arene ring *cis*-dihydroxylation, which yielded dihydrodiols of >98% ee. In contrast, NDO catalyzed exclusive formation of alkenadiols of (1*R*)-absolute configuration. With four of the seven substrates, the alkanediols were observed to accompany the ring dihydroxylation products formed by TDO [76]. The conversion of styrene to (*R*)-1-phenyl-1,2-ethanediol has been demonstrated with purified NDO [46,90]. In a separate example, TDO-catalyzed dihydroxylation and benzylic monohydroxylation of 2-methylindene yielding enantiopure (1*S*, 2*R*)-dihydroxy-2*S*-methylindan (26% yield), small amounts of (1*R*)-2-methylinden-1-ol (~1%) and the isomerization product 2-methyl-1-indanone (~57%), while NDO preferentially catalyzed monohydroxylation at the benzylic center yielding (1*S*)-2-methylinden-1-ol (67%, >98% ee) without isomerization to the ketone. Allylic hydroxylation yielded 2-hydroxy-methylindene as a minor product (5%). These trends observed for the absolute configuration of the mono- and dihydroxylation products formed by TDO and NDO were consistent with those previously reported for the enzymes with related benzocyclic substrates [47,53,54,87,91,92].

Chlorobenzene dioxygenase from *Pseudomonas* sp. strain P51 was initially noted for its ability to oxidize 1,2-dichlorobenzene, naphthalene, and a wide range of substituted benzenes, toluenes, biphenyls, and 3-ring aromatic *O*-heterocyclic compounds [17,93]. Chlorobenzene dioxygenase has also been developed as an efficient recombinant biocatalyst for *cis*-dihydroxylation of benzyl cyanide, cinnamitrile and a range of substituted benzonitriles; the enantiomeric purities of the *cis*-dihydroxylated products ranged from 43 to 97% ee [94].

Although crystal structures of several aromatic hydrocarbon dioxygenases are now available [67,95–99], the prediction of enzyme substrate specificity based on sequence or structure data is not yet reliable. The most useful approaches to identify an enzyme capable of catalyzing a desired reaction still remain (1) characterization of dioxygenase specificity profiles using diagnostic substrates, and (2) screening different enzymes on particular substrates for the desired target reaction or products.

10.4 BIOCATALYSIS STRATEGIES

Several properties associated with aromatic-ring-hydroxylating dioxygenases pose challenges for their application to the targeted preparation of oxidation products. These include their cofactor requirements, multicomponent nature, and low-specific activity and stability. The requirement for reduced nicotinamide cofactor(s) (NAD(P)H) combined with the multicomponent nature of the iron–sulfur containing subunits demand that an intricate balance be met to maintain enzyme stability and sustained productivity for the oxygenase-catalyzed reactions. Many monooxygenase and dioxygenase reactions have been demonstrated using purified oxygenase components with reduced cofactor supplied either through direct addition (stoichiometric amounts) or via cofactor regeneration systems. In these cases, the specific activities of purified enzymes are typically considered low (nmol/min/mg) when compared with other single component, cofactor-independent enzymes such as hydrolases (μmol/min/mg), which have been widely applied for bioreolution of pharmaceutical intermediates. As a result, applications based on aromatic-ring-hydroxylating dioxygenases have been based largely on whole-cell biotransformations typically with enzymes overexpressed in recombinant hosts such as *Escherichia coli*.

10.4.1 WHOLE-CELL BIOPROCESSES

Due to the cofactor requirements and increased efforts associated with the use of purified enzyme components or cell-free preparations, biocatalytic applications employing aromatic-ring-hydroxylating dioxygenases have been predominantly developed using whole-cell

systems. These whole-cell biotransformation systems are typically facilitated by the inducible overexpression of multicomponent dioxygenases in recombinant host strains, such as *E. coli* or *Pseudomonas* sp., which can be grown to high cell densities. These approaches have been employed for the application of oxygenases in bioprocesses attaining reasonably high volumetric yields [74,100,101].

10.4.2 COFACTOR REGENERATION

The whole-cell or resting-cell biotransformation approach has facilitated the production of multikilogram quantities of chiral metabolites and relies on the integrity of multicomponent dioxygenase activity, typically expressed in recombinant hosts with reduced cofactors supplied through the metabolism of exogenous carbon substrates (e.g., glucose, glycerol, pyruvate). A number of elegant approaches have been developed to enable biocatalysis with oxygenases in cell-free systems. The use of purified oxygenases can be facilitated by including systems for enzymatic [102] or electrochemical [103] regeneration of reduced NAD(P)H cofactor. The direct regeneration of flavin-dependent monooxygenases has also been demonstrated through the use of an organometallic redox complex [104]. The most common methods for enzymatic regeneration of nicotinamide coenzyme include formate dehydrogenase (FDH) for NAD(P)H [102,105], alcohol dehydrogenase [106], and glucose-6-phosphate dehydrogenase for NADPH [107]. FDH has been the most commonly utilized enzymatic regeneration system owing to its formation of carbon dioxide and specificity for either NADH or NADPH recycle. Examples of oxidoreductases utilized with enzyme-coupled cofactor recycle include 2-hydroxybiphenyl monooxygenase [102,108], styrene monooxygenase [109], and cyclohexanone monooxygenase [106].

Indirect electrochemical regeneration of NADH was employed with the flavin-dependent 2-hydroxybiphenyl-3-monooxygenase to give unoptimized rates that were ~50% of the *in vivo* process [103]. Recently, direct chemical regeneration was achieved for the FADH₂-dependent styrene monooxygenase (StyA) utilizing formate and a nonnative organometallic redox catalyst [104]. Initial productivities for styrene monooxygenase-catalyzed epoxidation of indene were compared with fully enzymatic (FDH) and chemoenzymatic regeneration of FADH₂. Direct regeneration systems were also demonstrated to replace NAD(P)H for several heme-containing P450 monooxygenases. Although not demonstrated for ring hydroxylating dioxygenases, the direct (electro)chemical regeneration offers potential for “cofactor-free” chemoenzymatic biocatalysis using isolated oxygenase enzymes for chemical synthesis.

10.4.3 REACTION AND PROCESS ENGINEERING APPROACHES

The oxygen-dependent alcohol oxidation reaction catalyzed by purified NDO was initially demonstrated by the oxidation of benzyl alcohol to benzaldehyde and 1-phenylethyl alcohol to acetophenone (the corresponding aldehydes were also observed as products from nitrotoluenes and xylenes) [46]. This reaction was also catalyzed with benzyl alcohol and 3,4-dimethylbenzyl alcohol using recombinant xylene monooxygenase, XylMA, expressed in *E. coli* [110]. Xylene monooxygenase catalyzed the multistep oxidation of one methyl group of toluenes, xylenes, and pseudocumene to corresponding alcohols, aldehydes, and acids; and has been used to develop aqueous-organic two-liquid phase (ATP) systems to exploit kinetics and control product formation in multistep biooxidations [111,112]. The application of recombinant XylMA-based whole-cell catalysis in ATP systems with fed-batch cultivation, using *bis*-(2-ethylhexyl)phthalate as carrier solvent, has been scaled to 30 L for the preparative conversion of pseudocumene to 3,4-dimethylbenzaldehyde at a concentration of 37 g/L; the product was isolated in 97% purity and 65% overall yield [113]. The ATP system has obvious

advantages for the regulation of concentrations (and associated toxicity) of apolar substrates and products in the aqueous microenvironment for the control of product formation and for facilitating product recovery [112]. Many aspects critical to the successful implementation of oxidative biocatalysis (e.g., high enzyme activity and specificity, product degradation, cofactor recycling, reactant toxicity, and substrate and oxygen mass transfer) can be addressed through biocatalyst and bioprocess engineering approaches, which have been recently reviewed [100].

10.4.4 BIOCATALYST IMPROVEMENT

Enzymes with new or improved activity or stability have been developed by a variety of laboratory engineering approaches, including “rational design” based on available or modeled crystal structures, saturation mutagenesis, error-prone PCR, and directed evolution of enzymes using DNA shuffling [114–122]. A promising new mutagenesis method, “combinatorial active-site saturations test” (CAST), involves saturation mutagenesis of spatially close pairs of amino acids at the active site, to allow more extensive active site reshaping with relatively small enzyme libraries [123]. Examples of the use of some of these methods to modify oxygenases follow.

Site-directed mutagenesis of amino acids located near the hydrophobic active site pocket of NDO from *Pseudomonas* sp. NCIB 9816-4 demonstrated that the enzyme was able to tolerate a wide range of single amino acid substitutions near the active site [124,125]. Enzymes with substitutions at position 352 of the oxygenase α subunit (phenylalanine in the wild-type enzyme) had altered enantioselectivity with naphthalene, biphenyl, phenanthrene and anthracene, and changes in the regioselectivity with biphenyl and phenanthrene [124,125]. For example, replacement of Phe352 with smaller amino acids (Gly, Ala, Val, Ile, Leu, and Thr) resulted in enzymes that produced increased amounts of biphenyl *cis*-3,4-dihydrodiol relative to biphenyl *cis*-2,3-dihydrodiol. In addition, the NDO-F352V and NDO-F352T enzymes formed significant amounts of (-)-biphenyl *cis*-(3*S*, 4*R*)-dihydrodiol, a compound not produced by wild-type NDO. Enzymes with substitutions at position 206 (NDO-A206I and NDO-A206I/L253T) formed significantly more phenanthrene *cis*-1,2-dihydrodiol than wild type, and several of the enzymes formed phenanthrene *cis*-9,10-dihydrodiol, a product not formed by the wild type [126].

In addition to the reported role of Phe352 in NDO from *Pseudomonas* sp. NCIB 9816-4 [124,125], substitution of the corresponding residue (Phe350) in NDO from *Ralstonia* sp. U2 with a threonine resulted in an enzyme with the ability to remove nitrite from 2,3- and 2,6-dinitrotoluene, but not from 2,4-dinitrotoluene [127]. Similarly, substitution of Val350 with either Phe or Met in the 2,4-dinitrotoluene dioxygenase from *Burkholderia cepacia* R34 resulted in enzymes with improved activities with substituted phenols [128]. Changing the corresponding residues in *B. xenovorans* LB400 BPDO and *Ralstonia* sp. PC12 tetrachlorobenzene dioxygenase (TecA) α subunits (Phe378 and Phe366, respectively) resulted in a reduction in overall activity, a change in the regiospecificity with chlorobiphenyl substrates for BPDO [129], and an increased preference for methyl group monooxygenation with mono- and dichlorotoluenes by TecA [130]. TecA also had a slightly increased preference for ring attack on 2,4,5-trichlorotoluene [130]. Substitution of tyrosine or tryptophan at this position either in TecA or in NDO resulted in inactive enzymes [125,130].

In another site-directed mutagenesis study, Phe227, Ile335, Thr376, and Phe377 in the α subunit of BPDO from *P. pseudoalcaligenes* KF707 were found to be important for determining the position of oxidation with various polychlorinated biphenyl (PCB) congeners [131]. Previous studies also demonstrated that Thr376 played an important role in determining regiospecificity with PCBs [132,133]. A variant large subunit biphenyl dioxygenase gene,

bphA, was evolved by DNA shuffling of the corresponding genes from *P. pseudoalcaligenes* KF707 and *B. xenovorans* LB400 [134]. The amino acid sequence of the evolved BphA differed by 4 and 15 amino acids when compared with those of KF707 and LB400, respectively [134]. When expressed in *E. coli*, this variant showed extended substrate specificity in its ability to dihydroxylate several phenyl-substituted heterocyclic compounds that were not substrates for the parent strains; the new substrates included 1-phenylpyrazole, 2-phenylpyridine, 4-phenylpyrimidine, 1-benzylimidazole, 4-phenylisothiazole, 2-benzylpyridine, and several other heterocyclic aromatic compounds [134].

Directed evolution was conducted with TDO to obtain variants that produced low amounts of the indene by-products (1-indenol and 1-indenone), while maintaining dihydroxylation activity to yield (-)-*cis*-(1*S*, 2*R*)-indandiol in high ee [135]. Three rounds of mutagenesis yielded variants producing more *cis*-indandiol relative to the indenol by-product, but enantioselectivity was altered to favor the production of the undesired (+)-*cis*-indandiol enantiomer [135] (see Section 5.3).

10.5 APPLICATIONS

Aromatic hydrocarbon dioxygenases have proven useful in a number of biotechnology applications. While they catalyze an impressive array of different reaction types, the stereoselective *cis*-dihydroxylation of nonactivated aromatic compounds is unique to this enzyme class and has been the basis for most applications. Examples include dioxygenase-catalyzed synthesis of chiral intermediates for the preparation of natural products, polyfunctionalized metabolites, and pharmaceutical intermediates; expression of recombinant NDO in an engineered bacterial strain for the production of indigo from glucose; and target-specific biodegradation of environmental pollutants.

10.5.1 ENANTIOSELECTIVE SYNTHESIS USING DIOXYGENASE-DERIVED SYNTHONS

Dioxygenase-derived chiral metabolites have been utilized in a variety of multistep syntheses for the preparation of fine chemicals, natural products, pharmaceutical intermediates, and biologically active compounds (Figure 10.2). The use of enzymatically formed *cis*-diols in enantioselective synthesis has been the subject of a comprehensive review [74] detailing synthetic design rationales, listing *cis*-diols accessible via dioxygenase-catalyzed *cis*-dihydroxylation and their application in asymmetric methodology for the synthesis of a wide range of cyclitols, conduritols, conduramines, inositols, heteroatom carbohydrates, alkaloids, and a variety of natural products. The *cis*-dihydrodiols of dictamnine and 4-chlorofuroquinoline yielded phenolic derivatives from which a range of furoquinoline alkaloids were synthesized [136]. A summary of recent progress in the synthesis of morphine alkaloids included the use of several metabolites derived via dioxygenase biocatalysis. Cyclohexadiene *cis*-dihydrodiols of phenethyl bromide and bromobenzene, as well as 3-bromocatechol (produced by a strain overexpressing TDO and DDH), have been employed as synthons in two separated synthetic strategies to produce advanced intermediates for the synthesis of morphine alkaloids [137]. The biooxidation of 4-bromoanisole by recombinant *E. coli* expressing TDO and DDH yielded *p*-methoxybromocatechol. This functionalized catechol was coupled with trimethoxyphenylacetylene in convergent syntheses of combretastatins A-1 and B-1, members of a class of oxygenated natural products with potent cytotoxic activity [138]. An efficient chemoenzymatic synthesis of strawberry furanone (4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one), a naturally occurring flavor compound, was enabled through directed evolution of TDO and tetrachlorobenzene dioxygenase operons that yielded improved enzymes for the conversion of *p*-xylene to the requisite diol synthon, *cis*-1,2-dihydroxy-3,6-dimethyl-3,5-cyclohexadiene (Figure 10.3a) [139].

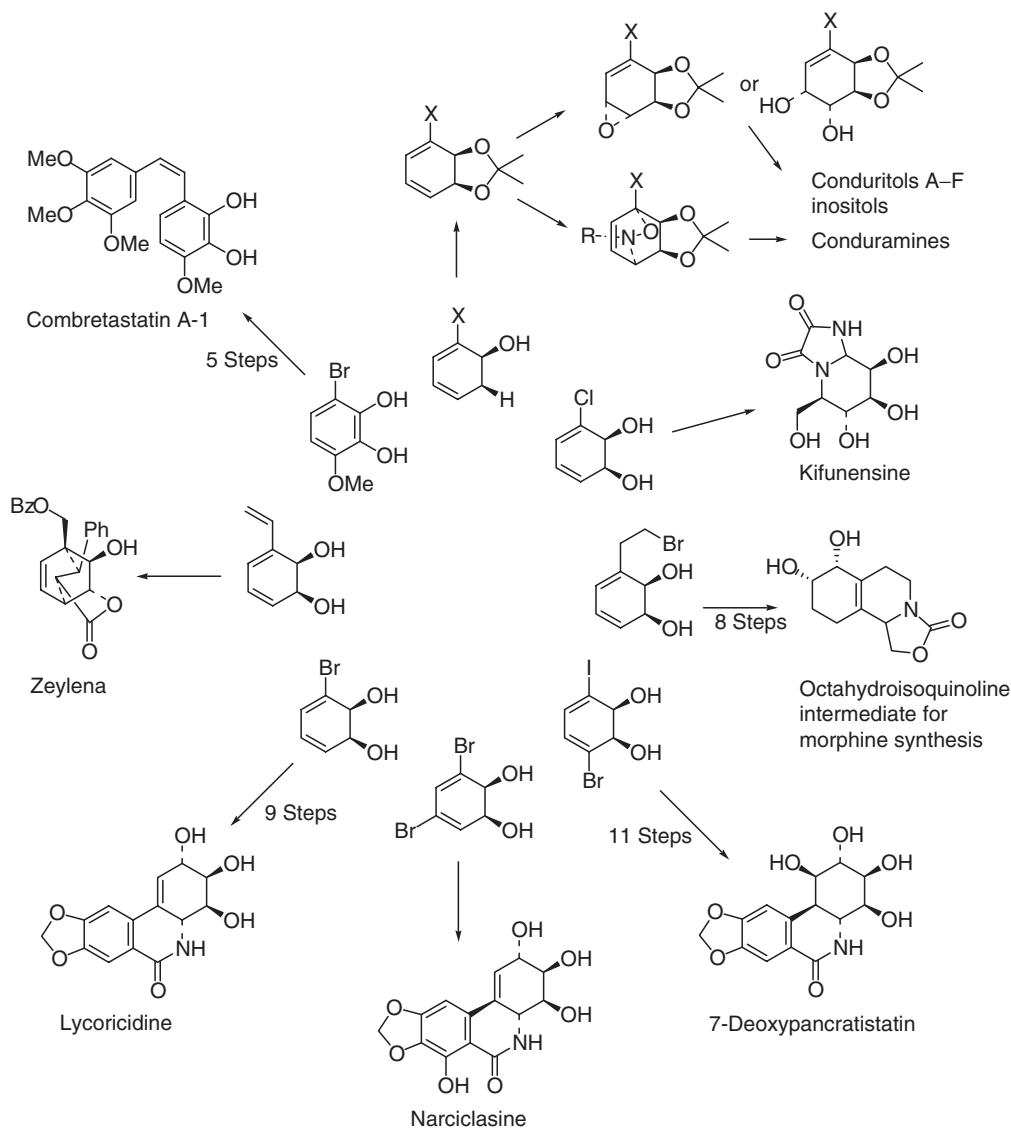


FIGURE 10.2 Examples of natural products, natural product intermediates, and analogs prepared via chemoenzymatic synthesis from the aromatic hydrocarbon dioxygenase-derived *cis*-dihydrodiols or catechols shown in the center. Hudlicky and coworkers provide an extensive review of specific synthetic applications to products from *cis*-dihydroarenediols. (From Hudlicky, T., Gonzalez, D., and Gibson, D.T., *Aldrichimica Acta*, 32, 35–62, 1999.)

10.5.2 DIOXYGENASE-CATALYZED REACTIONS FOR LEAD DEVELOPMENT AND NATURAL PRODUCT MODIFICATION

While *cis*-dihydrodiol metabolites, and in some cases *bis-cis*-diols [81,83,84], have been isolated from PAHs, benzo- and carbocyclic alkenes and polyenes, azaarenes, quinolines, and other heterocyclic aromatic compounds, the bacterial *cis*-dihydroxylation of an alkaloid natural product was reported only recently. Biotransformation of the parent furoquinoline alkaloid dictamine by BPDO in *S. yanoikuyae* strain B8/36 yielded the first isolable alkaloid *cis*-dihydrodiol

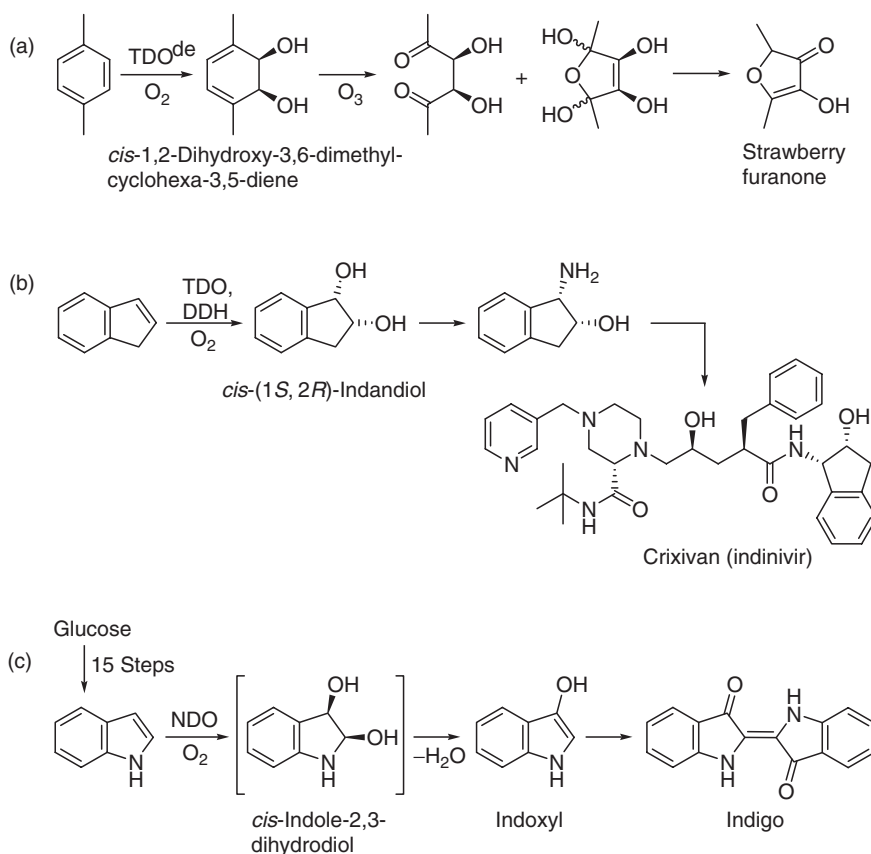


FIGURE 10.3 Biocatalytic processes developed based on aromatic hydrocarbon dioxygenases. (a) Preparation of the flavor compound strawberry furanone (4-hydroxy-2,5-dimethyl-2,3-dihydrofuran-3-one) via enzymatic dioxygenation of *p*-xylene by a variant of TDO obtained by directed evolution (TDO^{de}). (b) TDO-catalyzed dihydroxylation of indene and DDH-based resolution to (*cis*)-(1*S*, 2*R*)-indandiol preparation of indinivir. (c) NDO-catalyzed conversion of indole to indigo in a recombinant *E. coli* (glucose-fed) fermentation process.

metabolites [136]; dictamnine and its synthetic precursor, 4-chlorofuroquinoline, were substrates for asymmetric dihydroxylation each yielding two enantiopure *cis*-dihydrodiol regioisomers. Absolute configuration of the (7*S*, 8*R*)- and (5*R*, 6*S*)-*cis*-dihydrodiols, correlated through ¹H-NMR analysis of diastereomeric boronates prepared with (*R*)- and (*S*)-2-(1-methoxyethyl)-phenylboronic acid [140] and comparison of CD spectra, was consistent with those established for the *cis*-dihydrodiols formed from quinoline and acridines [84,141]. Small quantities of acyclic diols of variable enantiopurity were also observed and presumed to result from BPDO-catalyzed dihydroxylation of the dictamnine furan ring yielding a dihydrodiol undergoing spontaneous reversible ring-opening to the aldehyde that is enzymatically reduced to the observed diol. A similar metabolic sequence [furan \rightarrow furan *cis*-diol \leftrightarrow aldehyde \leftrightarrow furan *trans*-diol \rightarrow acyclic diol] observed during dihydroxylation of benzofuran [142] and benzothiophene [143] involves an equilibrium mixture of *cis/trans* diols and would result in the variable enantiopurity of the isolated acyclic diol. This approach illustrates the potential use of dioxygenase-catalyzed *cis*-dihydroxylation to access and expand the chemical diversity of not only plant alkaloids but also other natural products; such metabolites may be of considerable value for lead modification and oxyfunctionalization to afford synthons useful in the preparation of complex molecules with interesting biological activities.

10.5.3 INDINAVIR PRODUCTION

Biocatalytic production of enantiopure (-)-*cis*-(1*S*, 2*R*)-indandiol is of interest due to the availability of a reaction allowing direct conversion to *cis*-(1*S*)-amino-(2*R*)-indanol (Figure 10.3B), which is a key intermediate in the chemical synthesis of Merck's HIV-1 protease inhibitor Indinivir Sulfate (Crixivan) [144]. Whole-cell biotransformations conducted with toluene-induced *P. putida* F39/D (a mutant strain lacking DDH activity) or a recombinant TDO expressed in *E. coli* demonstrated that wild-type TDO oxidized indene to (-)-*cis*-(1*S*, 2*R*)-indandiol (~30% ee) and (1*R*)-indenol as the main products, with traces of 1-indenone formed [53,145]. However, the (-)-*cis*-(1*S*, 2*R*)-indandiol was obtained in >98% ee in the late stages of indene conversion with wild-type *P. putida* F1 [144] or by coexpression of DDH together with TDO in *E. coli* [145]. The increased enantiopurity of (-)-*cis*-(1*S*, 2*R*)-indandiol was found to occur at the expense of total indandiol yield as a result of kinetic resolution catalyzed by DDH that is selective for the undesired (+)-*cis*-(1*R*, 2*S*)-indandiol [145]. Directed evolution was used to select the variants of TDO that produced reduced amounts of the indene by-products 1-indenol and 1-indenone, while maintaining high (-)-*cis*-(1*S*, 2*R*)-indandiol enantiopurity [135]. After three rounds of mutagenesis, variants that produced significantly more *cis*-indandiol relative to the undesired by-product indenol were obtained. However, stereoselectivity was altered to favor the production of the undesired (+)-*cis*-indandiol enantiomer [135]. Neither these strategies nor the application of oxygenases from *Rhodococcus* strains [146] eliminated formation of indene by-products, and the maximum yields were limited to <60% (-)-*cis*-(1*S*, 2*R*)-indandiol. An alternative route to the vicinal aminoindanol involved the TDO-catalyzed enantioselective monohydroxylation of 2-indanol to (-)-*cis*-(1*S*, 2*R*)-indandiol. This reaction served as the basis for a process to prepare chiral 1-hydroxy-2-substituted indan intermediates [147]. *P. putida* strains UV-4 [148] and F39/D expressing TDO oxidized 2-indenol to (-)-*cis*-(1*S*, 2*R*)-indandiol in >98% ee and >85% yield; minor products included *trans*-1,2-indandiol (<15%) and 2-hydroxy-1-indanone (<2%) [149].

10.5.4 INDIGO PRODUCTION

The oxidation of indole to indigo was first shown in recombinant *E. coli* strains expressing NDO from *Pseudomonas* sp. NCIB 9816-4 [11]. NDO oxidized indole to an unstable *cis*-dihydroindole-2,3-diol that dehydrates to indoxyl, and subsequently undergoes spontaneous oxidation to indigo. This reaction is catalyzed by many related dioxygenases, and this simple colorimetric test has been widely utilized for detection and isolation of strains expressing mono- and dioxygenases, and in screening for mutants of these strains. Commercial interest in the reaction led Genencor International to genetically engineer a cost-competitive, multistep pathway for the production of indigo from glucose in *E. coli* (Figure 10.3C) [101]. The process for indigo production was based on a recombinant *E. coli* strain in which the tryptophan pathway was modified to allow a high level of indole production and cloned NDO from *P. putida* was expressed [101]. Numerous modifications were made to the strain to improve metabolite flux, eliminate the formation of the by-product isatin, and ultimately increase the production of indigo to levels exceeding 18 g/L. Despite the technical success of the process, the commercial production of indigo has not been implemented at an industrial scale.

10.5.5 BIOREMEDIATION OF TCE CONTAMINATION

Trichloroethylene (TCE) is widely used as a solvent and is classified as a priority pollutant by the U.S. Environmental Protection Agency. TCE is difficult to degrade and some of its breakdown products are actually more toxic than TCE itself. TDO from *P. putida* F1 and other di- and monooxygenases are capable of oxidizing TCE [150–152], but at this time, no

bacteria are known to utilize TCE as a carbon and energy source. TDO converts TCE into the nontoxic products formate and glyoxylate [153], and a hybrid TDO–BPDO was found to have enhanced TCE-degrading activity [154]. The purified hybrid protein was shown to have higher catalytic efficiency and a lower K_m for TCE than wild-type TDO [152]. Although TCE functions as an inducer of the genes encoding TDO in *P. putida* F1 [155], resting cultures exposed to TCE rapidly lose TCE oxidation activity [150]. A recent study demonstrated that the addition of benzene or toluene restored TCE-degrading activity to *P. putida* F1 cells [156], suggesting that TCE degradation could be improved with this strategy. A field trial monitoring TCE cooxidation suggested that this type of *in situ* bioremediation strategy might be feasible. In this study, toluene or phenol, and oxygen or hydrogen peroxide were added as cosubstrates to stimulate TCE cooxidation by indigenous bacteria, and >90% of the TCE, *cis*-dichloroethylene, and vinyl chloride were removed [157].

10.5.6 CONSTRUCTION OF NEW BIODEGRADATION PATHWAYS

Aromatic hydrocarbon dioxygenases, such as TDO, have been used to engineer new pathways for the degradation of recalcitrant compounds. Genes encoding TDO from *P. putida* F1 and cytochrome P450cam monooxygenase were used to construct a *Pseudomonas* strain able to metabolize polyhalogenated compounds by sequential reductive and oxidative reactions. In this engineered pathway, cytochrome P450cam catalyzes the conversion of highly chlorinated alkanes, such as pentachloroethane, to TCE under low oxygen tension, and TDO oxidizes TCE to glyoxalate and formate [158]. This type of hybrid pathway might find application in the clean up of sites contaminated with mixtures of polyhalogenated ethanes.

The *tod* genes encoding TDO from *P. putida* F1 were also cloned into the genome of *Deinococcus radiodurans*, an extremely radiation resistant bacterium. This recombinant strain was able to degrade toluene and related aromatic hydrocarbons in the presence of high levels of radiation [159]. Expression of the mercury resistance gene (*merA*) together with the TDO genes in *Deinococcus radiodurans* resulted in an engineered strain with the ability to remediate mixed radioactive waste containing aromatic hydrocarbon pollutants and the heavy metal mercury [160].

Many bacterial isolates are capable of degrading only a limited number of aromatic hydrocarbon pollutants, and genetic engineering has been used to increase the substrate range of specific microorganisms. When a constructed cassette, carrying genes for the conversion of styrene to phenylacetate, was introduced into *P. putida* F1 carrying the TOL plasmid, the engineered strain was capable of growth on an expanded range of aromatic hydrocarbons, including benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene, and styrene [161]. To reduce the undesirable possibility of horizontal transfer of genetically engineered DNA among bacteria in the environment, the cassette was introduced to a minitransposon carrying an engineered gene containment system [162]. Such a strain could prove useful in the bioremediation of low molecular weight aromatic hydrocarbon pollution.

10.6 OUTLOOK

We have tried to emphasize the versatility of aromatic-ring-hydroxylating dioxygenases with respect to the range of substrates attacked and the types of reactions catalyzed. Equally, if not more important, is the ability of many of these enzymes to form chiral products in high enantiomeric purity. To identify an enzyme that oxidizes a particular target and/or produces a specific product, one can attempt to isolate a new bacterial strain with the desired ability or screen the large number of well-characterized dioxygenases that are currently available. An alternative is to modify an available enzyme known to have a weak or similar activity by

random mutagenesis methods [116,163–165] or by rational design, taking advantage of the growing number of available dioxygenase crystal structures [67,95–99]. Finally, screening of metagenomic libraries [166,167], especially with samples from diverse environments, may allow the identification of new dioxygenases with useful activities without the need to isolate and characterize the host bacterium.

Once an enzyme with the desired selectivity is obtained, numerous steps are still required to develop viable and economical commercial processes. Efficiency can be realized through high protein expression coupled with process development. Overall productivity may also be increased by improving the activity or thermostability of the enzyme, or by reducing product inhibition. Process design must take into consideration the multicomponent nature of the enzyme and the NADH requirement when determining whether to use purified enzymes or whole-cells. In spite of the multiple challenges involved in using multicomponent dioxygenases, this class of enzymes holds significant promise for the development of environmentally friendly processes for reactions that are difficult, inefficient, or otherwise challenging to carry out by standard chemical methods.

ACKNOWLEDGMENTS

We thank David Gibson for critical review of the manuscript and Juan Parales for preparing Table 10.1. Research in the Parales laboratory is supported by the Army Research Office, the Strategic Environmental Research and Development Program, and the University of California Toxic Substances Research and Teaching Program.

REFERENCES

1. Gibson, D.T. and Parales, R.E., Aromatic hydrocarbon dioxygenases in environmental biotechnology, *Curr. Opin. Biotechnol.*, 11, 236–243, 2000.
2. Butler, C.S. and Mason, J.R., Structure–function analysis of the bacterial aromatic ring-hydroxylating dioxygenases, *Adv. Microbial. Physiol.*, 38, 47–84, 1997.
3. Yeh, W.-K., Gibson, D.T., and Liu, T.-N., Toluene dioxygenase: a multicomponent enzyme system, *Biochem. Biophys. Res. Commun.*, 78, 401–410, 1977.
4. Subramanian, V., Liu, T.-N., Yeh, W.-K., and Gibson, D.T., Toluene dioxygenase: purification of an iron-sulfur protein by affinity chromatography, *Biochem. Biophys. Res. Commun.*, 91, 1131–1139, 1979.
5. Gibson, D.T., Koch, J.R., and Kallio, R.E., Oxidative degradation of aromatic hydrocarbons by microorganisms I. Enzymatic formation of catechol from benzene, *Biochemistry*, 7, 2653–2661, 1968.
6. Kobal, V.M., Gibson, D.T., Davis, R.E., and Garza, A., X-ray determination of the absolute stereochemistry of the initial oxidation product formed from toluene by *Pseudomonas putida* 39/D, *J. Am. Chem. Soc.*, 95, 4420–4421, 1973.
7. Gibson, D.T., Hensley, M., Yoshioka, H., and Mabry, T.J., Formation of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*, *Biochemistry*, 9, 1626–1630, 1970.
8. Blumer, M., Polycyclic aromatic compounds in nature, *Sci. Am.*, 234, 35–41, 1976.
9. Gibson, D.T., Initial reactions to the bacterial degradation of aromatic hydrocarbons, *Zbl. Bakt. Hyg. I Abt. Orig. B.*, 162, 157–168, 1976.
10. Kiyohara, H., Nagao, K., and Yana, K., Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates, *Appl. Environ. Microbiol.*, 43, 454–457, 1982.
11. Ensley, B.D., Ratzkin, B.J., Osslund, T.D., Simon, M.J., Wackett, L.P., and Gibson, D.T., Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo, *Science*, 222, 167–169, 1983.
12. Eaton, R.W. and Chapman, P.J., Formation of indigo and related compounds from indolecarboxylic acids by aromatic acid-degrading bacteria: chromogenic reactions for cloning genes encoding dioxygenases that act on aromatic acids, *J. Bacteriol.*, 177, 6983–6988, 1995.

13. Kodama, K., Umehara, K., Shimizu, K., Nakatani, S., Minoda, Y., and Yamada, K., Identification of microbial products from dibenzothiophene and its proposed oxidation pathway, *Agric. Biol. Chem.*, 37, 45–50, 1973.
14. Kim, E. and Zylstra, G.J., Functional analysis of genes involved in biphenyl, naphthalene, phenanthrene, and *m*-xylene degradation by *Sphingomonas yanoikuyae* B1., *J. Ind. Microbiol. Biotechnol.*, 23, 294–302, 1999.
15. Zylstra, G.J., Kim, E., and Goyal, A.K., Comparative molecular analysis of genes for polycyclic aromatic hydrocarbon degradation, *Genet. Eng.*, 19, 257–269, 1997.
16. Haddock, J.D., Horton, J.R., and Gibson, D.T., Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400., *J. Bacteriol.*, 177, 20–26, 1995.
17. Werlen, C., Kohler, H.-P., and van der Meer, J.R., The broad substrate chlorobenzene dioxygenase and *cis*-chlorobenzene dihydrodiol dehydrogenase of *Pseudomonas* sp. strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation, *J. Biol. Chem.*, 271, 4009–4016, 1996.
18. Fetzner, S., Müller, R., and Lingens, F., Purification and some properties of 2-halobenzoate 1,2-dioxygenase, a two-component enzyme system from *Pseudomonas cepacia* 2CBS, *J. Bacteriol.*, 174, 279–290, 1992.
19. Nakatsu, C.H. and Wyndham, C., Cloning and expression of the transposable chlorobenzoate-3,4-dioxygenase genes of *Alcaligenes* sp. strain BR60, *Appl. Environ. Microbiol.*, 59, 3625–3633, 1993.
20. An, D., Gibson, D.T., and Spain, J.C., Oxidative release of nitrite from 2-nitrotoluene by a three-component enzyme system from *Pseudomonas* sp. strain JS42, *J. Bacteriol.*, 176, 7462–7467, 1994.
21. Spanggord, R.J., Spain, J.C., Nishino, S.F., and Mortelmans, K.E., Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp., *Appl. Environ. Microbiol.*, 57, 3200–3205, 1991.
22. Fukumori, F. and Saint, C., Nucleotide sequences and regulational analysis of genes involved in conversion of aniline to catechol in *Pseudomonas putida* UCC22(pTDN1), *J. Bacteriol.*, 179, 399–408, 1997.
23. Bundy, B.M., Campbell, A.L., and Neidle, E.L., Similarities between the *antABC*-encoded anthranilate dioxygenase and the *benABC*-encoded benzoate dioxygenase of *Acinetobacter* sp. strain ADP1, *J. Bacteriol.*, 180, 4466–4474, 1998.
24. Nishino, S.F. and Spain, J.C., Oxidative pathway for the biodegradation of nitrobenzene by *Comamonas* sp. strain JS765, *Appl. Environ. Microbiol.*, 61, 2308–2313, 1995.
25. Locher, H.H., Leisinger, T., and Cook, A.M., 4-Sulphobenzoate 3,4-dioxygenase: purification and properties of a desulphonative two-component enzyme system from *Comamonas testosteroni* T-2, *Biochem. J.*, 274, 833–842, 1991.
26. Engesser, K.H., Strubel, V., Christoglou, K., Fischer, P., and Rast, H.G., Dioxygenolytic cleavage of aryl ether bonds: 1,10-dihydro-1,10-dihydroxyfluoren-9-one, a novel arene dihydrodiol as evidence for angular dioxygenation of dibenzofuran, *FEMS Microbiol. Lett.*, 65, 205–210, 1989.
27. Bünz, P.V. and Cook, A.M., Dibenzofuran 4,4a-dioxygenase from *Sphingomonas* sp. strain RW1: angular dioxygenation by a three-component enzyme system, *J. Bacteriol.*, 175, 6467–6475, 1993.
28. Sato, S.-I., Nam, J.-W., Kasuga, K., Nojiri, H., Yamane, H., and Omori, T., Identification and characterization of genes encoding carbazole 1,9a-dioxygenase in *Pseudomonas* sp. strain CA10, *J. Bacteriol.*, 179(15), 4850–4858, 1997.
29. Engesser, K.H., Fietz, W., Fischer, P., Schulte, P., and Knackmuss, H.-J., Dioxygenolytic cleavage of aryl ether bonds: 1,2-dihydro-1,2-dihydroxy-4-carboxybenzophenone as evidence for initial 1,2-dioxygenation in 3- and 4-carboxy biphenyl ether degradation, *FEMS Microbiol. Lett.*, 69, 317–322, 1990.
30. Wittich, R.-M., Wilkes, H., Sinnwell, V., Francke, W., and Fortnagel, P., Metabolism of dibenzop-dioxin by *Sphingomonas* sp. strain RW1, *Appl. Environ. Microbiol.*, 58, 1005–1010, 1992.
31. Nojiri, H. and Omori, T., Molecular bases of aerobic bacterial degradation of dioxins: involvement of angular dioxygenation, *Biosci. Biotechnol. Biochem.*, 66, 2001–2016, 2002.
32. Nojiri, H., Habe, H., and Omori, T., Bacterial degradation of aromatic compounds via angular dioxygenation, *J. Gen. Appl. Microbiol.*, 47, 279–305, 2001.

33. Zhou, N.-Y., Al-Dulayymi, J., Baird, M.S., and Williams, P.A., Salicylate 5-hydroxylase from *Ralstonia* sp. strain U2: a monooxygenase with close relationships to and shared electron transport proteins with naphthalene dioxygenase, *J. Bacteriol.*, 184, 1547–1555, 2002.
34. Demaneche, S., Meyer, C., Micoud, J., Louwagie, M., Willison, J.C., and Jouanneau, Y., Identification and functional analysis of two aromatic ring-hydroxylating dioxygenases from a *Sphingomonas* strain that degrades various polycyclic aromatic hydrocarbons, *Appl. Environ. Microbiol.*, 70, 6714–6725, 2004.
35. Pinyakong, O., Habe, H., Yoshida, T., Nojiri, H., and Omori, T., Identification of three novel salicylate 1-hydroxylases involved in the phenanthrene degradation of *Sphingobium* sp. strain P2, *Biochem. Biophys. Res. Commun.*, 301, 350–357, 2003.
36. Cho, O., Choi, K.Y., Zylstra, G.J., Kim, Y.-S., Kim, S.-K., Lee, J.H., Sohn, H.-Y., Kwon, G.-S., Kim, Y.M., and Kim, E., Catabolic role of a three-component salicylate oxygenase from *Sphingomonas yanoikuyae* B1 in polycyclic aromatic hydrocarbon degradation, *Biochem. Biophys. Res. Commun.*, 327, 656–662, 2005.
37. Rosche, B., Tshisuaka, B., Fetzner, S., and Lingens, F., 2-Oxo-1,2-dihydroquinoline 8-monooxygenase, a two-component enzyme system from *Pseudomonas putida* 86, *J. Biol. Chem.*, 270, 17836–17842, 1995.
38. Junker, F., Kiewitz, R., and Cook, A.M., Characterization of the *p*-toluenesulfonate operon *tsaMBCD* and *tsaR* in *Comamonas testosteroni* T-2, *J. Bacteriol.*, 179(3), 919–927, 1997.
39. Bernhardt, F.-H., Erdin, N., Staudinger, H., and Ullrich, V., Interactions of substrates with a purified 4-methoxybenzoate monooxygenase system (*O*-demethylating) from *Pseudomonas putida*, *Eur. J. Biochem.*, 35, 126–134, 1973.
40. Venturi, V., Zennaro, F., Degrassi, G., Okeke, B.C., and Bruschi, C.V., Genetics of ferulic acid bioconversion to protocatechuic acid in plant-growth-promoting *Pseudomonas putida* WCS358, *Microbiology*, 144, 965–973, 1998.
41. Segura, A., Bunz, P.V., D'Argenio, D.A., and Ornston, L.N., Genetic analysis of a chromosomal region containing *vanA* and *vanB*, genes required for conversion of either ferulate or vanillate to protocatechuate in *Acinetobacter*, *J. Bacteriol.*, 181, 3439–3504, 1999.
42. Brunel, F. and Davison, J., Cloning and sequencing of *Pseudomonas* genes encoding vanillate demethylase, *J. Bacteriol.*, 170, 4924–4930, 1988.
43. Civolani, C., Barghini, P., Roncetti, A.R., Ruzzi, M., and Schiesser, A., Bioconversion of ferulic acid into vanillic acid by means of a vanillate-negative mutant of *Pseudomonas fluorescens* strain BF13, *Appl. Environ. Microbiol.*, 66, 2311–2317, 2000.
44. Priefert, H., Rabenhorst, J., and Steinbüchel, A., Molecular characterization of genes of *Pseudomonas* sp. strain HR199 involved in bioconversion of vanillin to protocatechuate, *J. Bacteriol.*, 179(8), 2595–2607, 1997.
45. Sonoki, T., Obi, T., Kubota, S., Higashi, M., Masai, E., and Katayama, Y., Coexistence of two different *O* demethylation systems in lignin metabolism by *Sphingomonas paucimobilis* SYK-6: cloning and sequencing of the lignin biphenyl-specific *O*-demethylase (LigX) gene., *Appl. Environ. Microbiol.*, 66, 2125–2132, 2000.
46. Lee, K. and Gibson, D.T., Toluene and ethylbenzene oxidation by purified naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4, *Appl. Environ. Microbiol.*, 62, 3101–3106, 1996.
47. Boyd, D.R., Sharma, N.D., Kerley, N.A., McMordie, R.A.S., Sheldrake, G.N., Williams, P., and Dalton, H., Dioxygenase-catalyzed oxidation of dihydronaphthalenes to yield arene hydrate and *cis*-dihydronaphthalenediols, *J. Chem. Soc. Perkin Trans. 1*, 67–74, 1996.
48. Resnick, S.M. and Gibson, D.T., Regio- and stereospecific oxidation of 9,10-dihydroanthracene and 9,10-dihydrophenanthrene by naphthalene dioxygenase: structure and absolute stereochemistry of metabolites, *Appl. Environ. Microbiol.*, 62, 3355–3359, 1996.
49. Selifonov, S.A., Grifoll, M., Eaton, R.W., and Chapman, P.J., Oxidation of naphthoaromatic and methyl-substituted aromatic compounds by naphthalene 1,2-dioxygenase, *Appl. Environ. Microbiol.*, 62, 507–514, 1996.
50. Resnick, S.M. and Gibson, D.T., Regio- and stereospecific oxidation of fluorene, dibenzofuran, and dibenzothiophene by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4, *Appl. Environ. Microbiol.*, 62, 4073–4080, 1996.

51. Brand, J.M., Cruden, D.L., Zylstra, G.J., and Gibson, D.T., Stereospecific hydroxylation of indan by *E. coli* containing the cloned toluene dioxygenase genes from *Pseudomonas putida* F1, *Appl. Environ. Microbiol.*, 58, 3407–3409, 1992.
52. Gibson, D.T., Resnick, S.M., Lee, K., Brand, J.M., Torok, D.S., Wackett, L.P., Schocken, M.J., and Haigler, B.E., Desaturation, dioxygenation and monooxygenation reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain 9816-4, *J. Bacteriol.*, 177, 2615–2621, 1995.
53. Wackett, L.P., Kwart, L.D., and Gibson, D.T., Benzylic monooxygenation catalyzed by toluene dioxygenase from *Pseudomonas putida*, *Biochemistry*, 27, 1360–1367, 1988.
54. Torok, D.S., Resnick, S.M., Brand, J.M., Cruden, D.L., and Gibson, D.T., Desaturation and oxygenation of 1,2-dihydronaphthalene by toluene and naphthalene dioxygenase, *J. Bacteriol.*, 177, 5799–5805, 1995.
55. Resnick, S.M. and Gibson, D.T., Biotransformation of anisole and phenetole by aerobic hydrocarbon-oxidizing bacteria, *Biodegradation*, 4, 195–203, 1993.
56. Nojiri, H., Nam, J.-W., Kosaka, M., Morii, K., Takemura, T., Furihata, K., Yamane, H., and Omori, T., Diverse oxygenations catalyzed by carbazole 1,9a-dioxygenase from *Pseudomonas* sp. strain CA10, *J. Bacteriol.*, 181, 3105–3113, 1999.
57. Allen, C.C.R., Boyd, D.R., Dalton, H., Sharma, N.D., Haughey, S.A., McMordie, R.A.S., McMurray, B.T., Sheldrake, G.N., and Sproule, K., Sulfoxides of high enantiopurity from bacterial dioxygenase-catalyzed oxidation, *J. Chem. Soc., Chem. Commun.*, 119–120, 1995.
58. Lee, K., Brand, J.M., and Gibson, D.T., Stereospecific sulfoxidation by toluene and naphthalene dioxygenases, *Biochem. Biophys. Res. Commun.*, 212, 9–15, 1995.
59. Lee, K., Biochemical studies on toluene and naphthalene dioxygenases, Ph.D. dissertation, University of Iowa, Iowa City, Iowa, 1995.
60. Resnick, S.M., Lee, K., and Gibson, D.T., Diverse reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816, *J. Ind. Microbiol.*, 17, 438–457, 1996.
61. Boyd, D.R., Sharma, N.D., Modyanova, L.V., Carroll, J.G., Malone, J.F., Allen, C.C.R., Hamilton, J.T.G., Gibson, D.T., Parales, R.E., and Dalton, H., Dioxygenase-catalyzed *cis*-dihydroxylation of pyridine-ring systems, *Can. J. Chem.*, 80, 589–600, 2002.
62. Haigler, B.E. and Gibson, D.T., Purification and properties of ferredoxin_{NAP}, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816, *J. Bacteriol.*, 172, 465–468, 1990.
63. Haigler, B.E. and Gibson, D.T., Purification and properties of NADH-ferredoxin_{NAP} reductase, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816, *J. Bacteriol.*, 172, 457–464, 1990.
64. Ensley, B.D. and Gibson, D.T., Naphthalene dioxygenase: purification and properties of a terminal oxygenase component, *J. Bacteriol.*, 155, 505–511, 1983.
65. Karlsson, A., Parales, J.V., Parales, R.E., Gibson, D.T., Eklund, H., and Ramaswamy, S., Crystal structure of naphthalene dioxygenase: side-on binding of dioxygen to iron, *Science*, 299, 1039–1043, 2003.
66. Simon, M.J., Osslund, T.D., Saunders, R., Ensley, B.D., Suggs, S., Harcourt, A., Suen, W.-C., Cruden, D.L., Gibson, D.T., and Zylstra, G.J., Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4, *Gene*, 127, 31–37, 1993.
67. Kauppi, B., Lee, K., Carredano, E., Parales, R.E., Gibson, D.T., Eklund, H., and Ramaswamy, S., Structure of an aromatic ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase, *Structure*, 6, 571–586, 1998.
68. Suen, W.-C. and Gibson, D.T., Isolation and preliminary characterization of the subunits of the terminal component of naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816-4, *J. Bacteriol.*, 175, 5877–5881, 1993.
69. Suen, W.-C. and Gibson, D.T., Recombinant *Escherichia coli* strains synthesize active forms of naphthalene dioxygenase and its individual α and β subunits, *Gene*, 143, 67–71, 1994.
70. Jiang, H., Parales, R.E., and Gibson, D.T., The α subunit of toluene dioxygenase from *Pseudomonas putida* F1 can accept electrons from reduced ferredoxin_{TOL} but is catalytically inactive in the absence of the β subunit, *Appl. Environ. Microbiol.*, 65, 315–318, 1999.

71. Hurtubise, Y., Barriault, D., and Sylvestre, M., Characterization of active recombinant His-tagged oxygenase component of *Comamonas testosteroni* B-356 biphenyl dioxygenase, *J. Biol. Chem.*, 271, 8152–8156, 1996.
72. Resnick, S.M., Torok, D.S., Lee, K., Brand, J.M., and Gibson, D.T., Regiospecific and stereoselective hydroxylation of 1-indanone and 2-indanone by naphthalene dioxygenase and toluene dioxygenase, *Appl. Environ. Microbiol.*, 60, 3323–3328, 1994.
73. Lange, C.C. and Wackett, L.P., Oxidation of aliphatic olefins by toluene dioxygenase: enzyme rates and product identification, *J. Bacteriol.*, 179, 3858–3865, 1997.
74. Hudlicky, T., Gonzalez, D., and Gibson, D.T., Enzymatic dihydroxylation of aromatics in enantioselective synthesis: expanding asymmetric methodology, *Aldrichimica Acta*, 32, 35–62, 1999.
75. Boyd, D.R. and Sheldrake, G.N., The dioxygenase-catalysed formation of vicinal *cis*-diols, *Nat. Prod. Rep.*, 15, 309–324, 1998.
76. Boyd, D.R., Sharma, N.D., Bowers, N.I., Brannigan, I.N., Groocock, M.R., Malone, J.F., McConville, G., and Allen, C.C.R., Biocatalytic asymmetric dihydroxylation of conjugated mono- and poly-alkenes to yield enantiopure cyclic *cis*-diols, *Adv. Synth. Catal.*, 347, 1081–1089, 2005.
77. Gibson, D.T., *Beijerinckia* sp. strain B1: a strain by any other name, *J. Ind. Microbiol. Biotechnol.*, 23, 284–293, 1999.
78. Schocken, M.J. and Gibson, D.T., Bacterial oxidation of the polycyclic aromatic hydrocarbons acenaphthene and acenaphthylene, *Appl. Environ. Microbiol.*, 48, 10–16, 1984.
79. Mahaffey, W.R., Gibson, D.T., and Cerniglia, C.E., Bacterial oxidation of chemical carcinogens: formation of polycyclic aromatic acids from benz[a]anthracene, *Appl. Environ. Microbiol.*, 54, 2415–2423, 1988.
80. Gibson, D.T., Roberts, R.L., Wells, M.C., and Kobal, V.M., Oxidation of biphenyl by a *Beijerinckia* species, *Biochem. Biophys. Res. Commun.*, 50, 211–219, 1973.
81. Boyd, D.R., Sharma, N.D., Agarwal, R., Resnick, S.M., Schocken, M.J., Gibson, D.T., Sayer, J.M., Yagi, H., and Jerina, D.M., Bacterial dioxygenase-catalyzed dihydroxylation and chemical resolution routes to enantiopure *cis*-dihydrodiols of chrysene, *J. Chem. Soc. Perkin Trans. 1*, 1715–1723, 1997.
82. Jerina, D.M., Van Bladeren, P.J., Yagi, H., Gibson, D.T., Mahadevan, V., Neese, A.S., Koreeda, M., Sharma, N.D., and Boyd, D.R., Synthesis and absolute configuration of the bacterial *cis*-1,2-, *cis*-8,9-, and *cis*-10,11-dihydrodiol metabolites of benz[a]anthracene formed by a strain of *Beijerinckia*, *J. Org. Chem.*, 49, 3621–3628, 1984.
83. Boyd, D.R., Sharma, N.D., Hempenstall, F., Kennedy, M.A., Malone, J.F., Allen, C.C.R., Resnick, S.M., and Gibson, D.T., *bis-cis*-Dihydrodiols: a new class of metabolites resulting from biphenyl dioxygenase-catalyzed sequential asymmetric *cis*-dihydroxylation of polycyclic arenes and heteroarenes, *J. Org. Chem.*, 64, 4005–4011, 1999.
84. Boyd, D.R., Sharma, N.D., Carroll, J.G., Allen, C.C.R., Clarke, D.A., and Gibson, D.T., Multiple site dioxygenase-catalysed *cis*-dihydroxylation of polycyclic azaarenes to yield a new class of *bis-cis*-diol metabolites, *Chem. Commun.*, 1201–1202, 1999.
85. Finn, K.J., Cankafi, P., Jones, T.R.B., and Hudlicky, T., Enzymatic oxidation of thioanisoles: isolation and absolute configuration of metabolites, *Tetrahedron Asymmetry*, 15, 2833–2836, 2004.
86. Bowers, N.I., Boyd, D.R., Sharma, N.D., Goodrich, P.A., Groocock, M.R., Blacker, A.J., Goode, P., and Dalton, H., Stereoselective benzylic hydroxylation of 2-substituted indanes using toluene dioxygenase as biocatalysts, *J. Chem. Soc. Perkin Trans. 1*, 1453–1461, 1999.
87. Lee, K., Resnick, S.M., and Gibson, D.T., Stereospecific oxidation of (*R*)- and (*S*)-indanol by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4, *Appl. Environ. Microbiol.*, 63, 2067–2070, 1997.
88. Resnick, S., Diverse reactions catalyzed by multicomponent aromatic hydrocarbon dioxygenases, Ph.D. thesis, University of Iowa, Iowa City, 1997.
89. Resnick, S.M. and Gibson, D.T., Oxidation of polycyclic aromatic compounds and formation of novel *cis*-diol enantiomers by carbazole utilizing pseudomonads, in *Abstract of the 96th General Meeting of the American Society for Microbiology*, New Orleans, LA, O-11, 1996.

90. Lee, K. and Gibson, D.T., Stereospecific dihydroxylation of the styrene vinyl group by purified naphthalene dioxygenase from *Pseudomonas* sp. strain 9816-4, *J. Bacteriol.*, 178, 3353–3356, 1996.
91. Resnick, S.M. and Gibson, D.T., Oxidation of 6,7-dihydro-5H-benzocycloheptene by bacterial strains expressing naphthalene dioxygenase, biphenyl dioxygenase, and toluene dioxygenase yields homochiral monol or *cis*-diol enantiomers as major products, *Appl. Environ. Microbiol.*, 62, 1364–1368, 1996.
92. Boyd, D.R., Dorrity, M.R.J., Malone, J.F., McMordie, R.A.S., Sharma, N.D., Dalton, H., and Williams, P., Bacterial metabolism of 6,7-dihydro-5H-benzocycloheptene by *Pseudomonas putida*. Synthesis and absolute configuration of benzylic alcohol and *cis*-diol metabolites of 6,7-dihydro-5H-benzocycloheptene, *J. Chem. Soc. Perkin. Trans. I*, 1990, 489–494, 1990.
93. Raschke, H., Meier, M., Burken, J.G., Hany, R., Muller, M.D., van der Meer, J.R., and Kohler, H.P., Biotransformation of various substituted aromatic compounds to chiral dihydrodihydroxy derivatives, *Appl. Environ. Microbiol.*, 67, 3333–3339, 2001.
94. Yildirim, S., Franco, T.T., Wohlgemuth, R., Kohler, H.-P.E., Witholt, B., and Schmid, A., Recombinant chlorobenzene dioxygenase from *Pseudomonas* sp. P51: a biocatalyst for regioselective oxidation of aromatic nitriles, *Adv. Synth. Catal.*, 347, 1060–1072, 2005.
95. Furusawa, Y., Nagarajan, V., Tanokura, M., Masai, E., Fukuda, M., and Senda, T., Crystal structure of the terminal oxygenase of biphenyl dioxygenase from *Rhodococcus* sp. strain RHA1, *J. Mol. Biol.*, 342, 1041–1052, 2004.
96. Friemann, R., Ivkovic-Jensen, M.M., Lessner, D.J., Yu, C.-L., Gibson, D.T., Parales, R.E., Eklund, H., and Ramaswamy, S., Structural insights into the dioxygenation of nitroarene compounds: the crystal structure of the nitrobenzene dioxygenase, *J. Mol. Biol.*, 348, 1139–1151, 2005.
97. Nojiri, H., Ashikawa, Y., Noguchi, H., Nam, J.-W., Urata, M., Fujimoto, Z., Uchimura, H., Terada, T., Nakamura, S., Shimizu, K., Yoshida, T., Habe, H., and Omori, T., Structure of the terminal oxygenase component of angular dioxygenase, carbazole 1,9a-dioxygenase, *J. Mol. Biol.*, 351, 355–370, 2005.
98. Dong, X., Fushinobu, S., Fukuda, E., Terada, T., Nakamura, S., Shimizu, K., Nojiri, H., Omori, T., Shoun, H., and Wakagi, T., Crystal structure of the terminal oxygenase component of cumene dioxygenase from *Pseudomonas fluorescens* IP01, *J. Bacteriol.*, 187, 2483–2490, 2005.
99. Martins, B.M., Svetlitchnaia, T., and Dobbek, H., 2-Oxoquinoline 8-monooxygenase oxygenase component: active site modulation by Rieske-[2Fe-2S] center oxidation/reduction, *Structure*, 13, 817–824, 2005.
100. Buhler, B. and Schmid, A., Process implementation aspects for biocatalytic hydrocarbon oxyfunctionalization, *J. Biotechnol.*, 113, 183–210, 2004.
101. Berry, A., Dodge, T.C., Pepsin, M., and Weyler, W., Application of metabolic engineering to improve both the production and use of biotech indigo, *J. Ind. Microbiol. Biotechnol.*, 28, 127–133, 2002.
102. Schmid, A., Vereyken, I., Held, M., and Witholt, B., Preparative regio- and chemoselective functionalization of hydrocarbons catalyzed by cell free preparations of 2-hydroxybiphenyl 3-monooxygenase, *J. Mol. Catal., B Enzym.*, 11, 455–462, 2001.
103. Hollmann, F., Schmid, A., and Steckhan, E., First synthetic application of a monooxygenase employing indirect electrochemical NADH regeneration, *Angew. Chem. Int. Ed.*, 40, 169–171, 2001.
104. Hollmann, F., Lin, P.-C., Witholt, B., and Schmid, A., Stereospecific biocatalytic epoxidation: the first example of direct regeneration of a FAD-dependent monooxygenase for catalysis, *J. Am. Chem. Soc.*, 125, 8209–8217, 2003.
105. Rissoma, S., Schwarz-Linekb, U., Vogelb, M., Tishkovc, V.I., and Kragl, U., Synthesis of chiral epsilon-lactones in a two-enzyme system of cyclohexanone mono-oxygenase and formate dehydrogenase with integrated bubble-free aeration, *Tetrahedron Asymmetry*, 8, 2523–2526, 1997.
106. Zambianchi, F., Pasta, P., Carrea, G., Colonna, S., Gaggero, N., and Woodley, J.M., Use of isolated cyclohexanone monooxygenase from recombinant *Escherichia coli* as a biocatalyst for Baeyer–Villiger and sulfide oxidations, *Biotechnol. Bioeng.*, 78, 489–496, 2002.
107. Wong, C.-H. and Whitesides, G.M., Enzyme-catalyzed organic synthesis: NAD(P)H cofactor regeneration by using glucose-6-phosphate and the glucose-5-phosphate dehydrogenase from *Leuconostoc mesenteroides*, *J. Am. Chem. Soc.*, 103, 4890–4899, 1981.

108. Lutz, J., Mozhaev, V.V., Khmelnitsky, Y.L., Witholt, B., and Schmid, A., Preparative application of 2-hydroxybiphenyl 3-monooxygenase with enzymatic cofactor regeneration in organic-aqueous reaction media, *J. Mol. Catal., B Enzym.*, 19/20, 177–187, 2002.
109. Hofstetter, K., Lutz, J., Lang, I., Witholt, B., and Schmid, A., Biocatalytic asymmetric epoxidation with NADH regeneration in organic-aqueous emulsions, *Angew. Chem. Int. Ed. Engl.*, 43, 2163–2166, 2004.
110. Buhler, B., Schmid, A., Hauer, B., and Witholt, B., Xylene monooxygenase catalyzes the multistep oxygenation of toluene and pseudocumene to corresponding alcohols, aldehydes, and acids in *Escherichia coli* JM101, *J. Biol. Chem.*, 275, 10085–10092, 2000.
111. Buhler, B., Witholt, B., Hauer, B., and Schmid, A., Characterization and application of xylene monooxygenase for multistep biocatalysis, *Appl. Environ. Microbiol.*, 68, 560–568, 2002.
112. Buhler, B., Bollhalder, I., Hauer, B., Witholt, B., and Schmid, A., Use of the two-liquid phase concept to exploit kinetically controlled multistep biocatalysis, *Biotechnol. Bioeng.*, 81, 683–694, 2003.
113. Buhler, B., Bollhalder, I., Hauer, B., Witholt, B., and Schmid, A., Chemical biotechnology for the specific oxyfunctionalization of hydrocarbons on a technical scale, *Biotechnol. Bioeng.*, 82, 833–842, 2003.
114. Harayama, S., Artificial evolution by DNA shuffling, *Trends Biotechnol.*, 16, 76–82, 1998.
115. Cramer, A., Raillard, S.-A., Bermudez, E., and Stemmer, W.P.C., DNA shuffling of a family of genes from diverse species accelerates directed evolution, *Nature*, 391, 288–291, 1998.
116. Cirino, P.C. and Arnold, F.H., Protein engineering of oxygenases for biocatalysis, *Curr. Opin. Chem. Biol.*, 6, 130–135, 2002.
117. Zhao, H. and Arnold, F.H., Optimization of DNA shuffling for high fidelity recombination, *Nucleic Acids Res.*, 6, 1307–1308, 1997.
118. Lutz, S. and Ostermeier, M., Preparation of SCRATCHY hybrid protein libraries: size- and in-frame selection of nucleic acid sequences, *Methods Mol. Biol.*, 231, 143–151, 2003.
119. Ostermeier, M. and Lutz, S., The creation of ITCHY hybrid protein libraries, *Methods Mol. Biol.*, 231, 129–141, 2003.
120. Cirino, P.C., Mayer, K.M., and Umeno, D., Generating mutant libraries using error-prone PCR, *Methods Mol. Biol.*, 231, 3–9, 2003.
121. Georgescu, R., Bandara, G., and Sun, L., Saturation mutagenesis, *Methods Mol. Biol.*, 231, 75–83, 2003.
122. Coco, W.M., RACHITT: gene family shuffling by random chimeragenesis on transient templates, *Methods Mol. Biol.*, 231, 111–127, 2003.
123. Reetz, M.T., Bocla, M., Carballeira, J.D., Zha, D., and Vogel, A., Expanding the range of substrate acceptance of enzymes: combinatorial active-site saturation test, *Angew. Chem. Int. Ed. Engl.*, 44, 4192–4196, 2005.
124. Parales, R.E., Lee, K., Resnick, S.M., Jiang, H., Lessner, D.J., and Gibson, D.T., Substrate specificity of naphthalene dioxygenase: effect of specific amino acids at the active site of the enzyme, *J. Bacteriol.*, 182, 1641–1649, 2000.
125. Parales, R.E., Resnick, S.M., Yu, C.L., Boyd, D.R., Sharma, N.D., and Gibson, D.T., Regioselectivity and enantioselectivity of naphthalene dioxygenase during arene *cis*-dihydroxylation: control by phenylalanine 352 in the α subunit, *J. Bacteriol.*, 182, 5495–5504, 2000.
126. Yu, C.-L., Parales, R.E., and Gibson, D.T., Multiple mutations at the active site of naphthalene dioxygenase affect regioselectivity and enantioselectivity, *J. Ind. Microbiol. Biotechnol.*, 27, 94–103, 2001.
127. Keenan, B.G., Leungsakul, T., Smets, B.F., Mori, M.A., Henderson, D.E., and Wood, T.K., Protein engineering of the archetypal nitroarene dioxygenase of *Ralstonia* sp. strain U2 for activity on aminonitrotoluenes and dinitrotoluenes through alpha-subunit residues leucine 225, phenylalanine 350, and glycine 407, *J. Bacteriol.*, 187, 3302–3310, 2005.
128. Keenan, B.G., Leungsakul, T., Smets, B.F., and Wood, T.K., Saturation mutagenesis of *Burkholderia cepacia* R34 2,4-dinitrotoluene dioxygenase at DntAc valine 350 for synthesizing nitrohydroquinone, methylhydroquinone, and methoxyhydroquinone, *Appl. Environ. Microbiol.*, 70, 3222–3231, 2004.

129. Zielinski, M., Kahl, S., Hecht, H.J., and Hofer, B., Pinpointing biphenyl dioxygenase residues that are crucial for substrate interaction, *J. Bacteriol.*, 185, 6976–6980, 2003.
130. Pollmann, K., Wray, V., Hecht, H.-J., and Pieper, D.H., Rational engineering of the regioselectivity of TecA tetrachlorobenzene dioxygenase for the transformation of chlorinated toluenes, *Microbiology*, 149, 903–913, 2003.
131. Suenaga, H., Watanabe, T., Sato, M., Ngadiman, and Furukawa, K., Alteration of regiospecificity in biphenyl dioxygenase by active-site engineering, *J. Bacteriol.*, 184, 3682–3688, 2002.
132. Mondello, F.J., Turcich, M.P., Lobos, J.H., and Erickson, B.D., Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation, *Appl. Environ. Microbiol.*, 63, 3096–3103, 1997.
133. Kimura, N., Nishi, A., Goto, M., and Furukawa, K., Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally, *J. Bacteriol.*, 179, 3936–3943, 1997.
134. Misawa, N., Shindo, K., Takahashi, H., Suenaga, H., Iguchi, K., Okazaki, H., Harayama, S., and Furukawa, K., Hydroxylation of various molecules including heterocyclic aromatics using recombinant *Escherichia coli* cells expressing modified biphenyl dioxygenase genes, *Tetrahedron*, 58, 9605–9612, 2002.
135. Zhang, N., Stewart, B.G., Moore, J.C., Greasham, R.L., Robinson, D.K., Buckland, B.C., and Lee, C., Directed evolution of toluene dioxygenase from *Pseudomonas putida* for improved selectivity toward *cis*-indandiol during indene bioconversion, *Metab. Eng.*, 2, 339–348, 2000.
136. Boyd, D.R., Sharma, N.D., O'Dowd, C.R., Carroll, J.G., Loke, P.L., and Allen, C.C.R., *cis*-Dihydrodiol, arene oxide and phenol metabolites of dictamnine: key intermediates in the biodegradation and biosynthesis of furoquinoline alkaloids, *Chem. Commun.*, 31, 3989–3991, 2005.
137. Zezula, J. and Hudlicky, T., Recent progress in the synthesis of morphine alkaloids, *Synlett*, 3, 388–405, 2005.
138. Bui, V.P., Hudlicky, T., Hansen, T.V., and Stenstrom, Y., Direct biooxidation of arenes to corresponding catechols with *E. coli* JM109(pDTG602). Application to synthesis of combretastatins A-1 and B-1, *Tetrahedron Lett.*, 43, 2839–2841, 2002.
139. Newman, L.M., Garcia, H., Hudlicky, T., and Selifonov, S.A., Directed evolution of the dioxygenase complex for the synthesis of furanone flavor compounds, *Tetrahedron*, 60, 729–734, 2004.
140. Resnick, S.M., Torok, D.S., and Gibson, D.T., Chemoenzymatic synthesis of chiral boronates for the ¹H NMR determination of the absolute configuration and enantiomeric excess of bacterial and synthetic *cis*-diols, *J. Org. Chem.*, 60, 3546–3549, 1995.
141. Boyd, D.R., Sharma, N.D., Dorrity, M.R.J., Hand, M.V., McMordie, R.A.S., Malone, J.F., Porter, H.P., Chima, J., Dalton, H., and Sheldrake, G.N., Structure and stereochemistry of *cis*-dihydrodiol and phenol metabolites of bicyclic azaarenes from *Pseudomonas putida* UV4, *J. Chem. Soc. Perkin Trans. 1*, 1065–1071, 1993.
142. Boyd, D.R., Sharma, N.D., Brannigan, I.N., Haughey, S.A., Malone, J.F., Clarke, D.A., and Dalton, H., Dioxygenase-catalysed formation of *cis/trans*-dihydrodiol metabolites of mono- and bi-cyclic heteroarenes, *Chem. Commun.*, 2361–2362, 1996.
143. Eaton, R.W. and Nitterauer, J.D., Biotransformation of benzothiophene by isopropylbenzene-degrading bacteria, *J. Bacteriol.*, 176, 3992–4002, 1994.
144. Connors, N., Prevost, R., Chartrain, M., Reddy, J., Singhvi, R., Patel, Z., Olewinski, R., Salmon, P., Wilson, J., and Greasham, R., Conversion of indene to *cis*-(1*S*, 2*R*)-indandiol by mutants of *Pseudomonas putida* F1, *J. Ind. Microbiol. Biotechnol.*, 18, 353–359, 1997.
145. Reddy, J., Lee, C., Nepper, M., Greasham, R., and Zhang, J., Development of a bioconversion process for production of *cis*-1*S*, 2*R*-indandiol from indene by recombinant *Escherichia coli* constructs, *Appl. Microbiol. Biotechnol.*, 51, 614–620, 1999.
146. O'Brien, X.M., Parker, J.A., Lessard, P.A., and Sinskey, A.J., Engineering an indene bioconversion process for the production of *cis*-aminoindanol: a model system for the production of chiral synthons, *Appl. Microbiol. Biotechnol.*, 59, 389–399, 2002.
147. Blacker, A.J., Boyd, D.R., Dalton, H., and Bowers, N., WO 96/37628, Preparation of hydroxyl compounds by conversion with dioxygenase. May 20, 1996.

148. Boyd, D.R., Sharma, N.D., Bowers, N.I., Goodrich, P.A., Groocock, M.R., Blaker, A.J., Clarke, D.A., Howard, T., and Dalton, H., Stereoselective dioxygenase-catalyzed benzylic hydroxylation at prochiral methylene groups in the chemoenzymatic synthesis of enantiopure vicinal aminoindanols, *Tetrahedron Asymmetry*, 7, 1559–1562, 1996.
149. Lakshman, M.K., Chaturvedi, S., Zajc, B., Gibson, D.T., and Resnick, S.M., A general chemoenzymatic synthesis of enantiopure *cis* β -amino alcohols from microbially derived *cis*-glycols, *Synthesis*, 9, 1352–1356, 1998.
150. Wackett, L.P. and Gibson, D.T., Degradation of trichloroethylene by toluene dioxygenase in whole cell studies with *Pseudomonas putida* F1, *Appl. Environ. Microbiol.*, 54, 1703–1708, 1988.
151. Kessler, M., Dabbs, E.R., Averhoff, B., and Gottschalk, G., Studies on the isopropylbenzene 2,3-dioxygenase and 3-isopropylcatechol 2,3-dioxygenase genes encoding by the linear plasmid of *Rhodococcus erythropolis* BD2, *Microbiology*, 142, 3241–3251, 1996.
152. Maeda, T., Takahashi, Y., Suenaga, H., Suyama, A., Goto, M., and Furukawa, K., Functional analyses of Bph-Tod hybrid dioxygenase, which exhibits high degradation activity toward trichloroethylene, *J. Biol. Chem.*, 276, 29833–29838, 2001.
153. Li, S. and Wackett, L.P., Trichloroethylene oxidation by toluene dioxygenase, *Biochem. Biophys. Res. Commun.*, 185, 443–451, 1992.
154. Furukawa, K., Hirose, J., Hayashida, S., and Nakamura, K., Efficient degradation of trichloroethylene by a hybrid aromatic ring dioxygenase, *J. Bacteriol.*, 176(7), 2121–2123, 1994.
155. Shingleton, J.T., Applegate, B.M., Nagel, A.C., Bienkowski, P.R., and Sayler, G.S., Induction of the *tod* operon by trichloroethylene in *Pseudomonas putida* TVA8, *Appl. Environ. Microbiol.*, 64, 5049–5052, 1998.
156. Morono, Y., Unno, H., Tanji, Y., and Hori, K., Addition of aromatic substrates restores trichloroethylene degradation activity in *Pseudomonas putida* F1, *Appl. Environ. Microbiol.*, 70, 2830–2835, 2004.
157. Hopkins, G.D. and McCarty, P.L., Field evaluation of *in-situ* aerobic cometabolism of trichloroethene and three dichloroethene isomers using phenol and toluene as the primary substrates, *Environ. Sci. Technol.*, 29, 1628–1637, 1995.
158. Wackett, L.P., Sadowsky, M.J., Newman, L.M., Hur, H.-G., and Li, S., Metabolism of polyhalogenated compounds by a genetically engineered bacterium, *Nature*, 368, 627–629, 1994.
159. Lange, C.C., Wackett, L.P., Minton, K.W., and Daly, M.J., Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments, *Nat. Biotechnol.*, 16, 929–933, 1998.
160. Brim, H., McFarlan, S.C., Fredrickson, J.K., Minton, K.W., Zhai, M., Wackett, L.P., and Daly, M.J., Engineering *Deinococcus radiodurans* for metal remediation in radioactive mixed waste environments, *Nat. Biotechnol.*, 18, 85–90, 2000.
161. Lorenzo, P., Alonso, S., Velasco, A., Diaz, E., Garcia, J.L., and Perera, J., Design of catabolic cassettes for styrene biodegradation, *Antonie Van Leeuwenhoek.*, 84, 17–24, 2003.
162. Diaz, E., Munthali, M., de Lorenzo, V., and Timmis, K.N., Universal barrier to lateral spread of specific genes among microorganisms, *Mol. Microbiol.*, 13, 855–861, 1994.
163. Jaeger, K.E. and Eggert, T., Enantioselective biocatalysis optimized by directed evolution, *Curr. Opin. Biotechnol.*, 15, 305–313, 2004.
164. Otten, L.G. and Quax, W.J., Directed evolution: selecting today's biocatalysts, *Biomol. Eng.*, 22, 1–9, 2005.
165. Zhao, H., Chockalingam, K., and Chen, Z., Directed evolution of enzymes and pathways for industrial biocatalysis, *Curr. Opin. Biotechnol.*, 13, 104–110, 2002.
166. Daniel, R., The soil metagenome—a rich resource for the discovery of novel natural products, *Curr. Opin. Biotechnol.*, 15, 199–204, 2004.
167. Lorenz, P., Liebeton, K., Niehaus, F., and Eck, J., Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space, *Curr. Opin. Biotechnol.*, 13, 572–577, 2002.
168. Jeon, C.O., Park, W., Padmanabhan, P., DeRito, C., Snape, J.R., and Madsen, E.L., Discovery of a bacterium, with distinctive dioxygenase, that is responsible for *in situ* biodegradation in contaminated sediment, *Proc. Natl. Acad. Sci. USA*, 100, 13591–13596, 2003.

169. Larkin, M.J., Allen, C.C.R., Kulakov, L.A., and Lipscomb, D.A., Purification and characterization of a novel naphthalene dioxygenase from *Rhodococcus* sp. strain NCIMB12038, *J. Bacteriol.*, 181, 6200–6204, 1999.
170. Denome, S.A., Stanley, D.C., Olsen, E.S., and Young, K.D., Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway, *J. Bacteriol.*, 175, 6890–6901, 1993.
171. Takizawa, N., Kaida, N., Torigoe, S., Moritani, T., Sawada, T., Satoh, S., and Kiyohara, H., Identification and characterization of genes encoding polycyclic aromatic hydrocarbon dioxygenase and polycyclic aromatic hydrocarbon dihydrodiol dehydrogenase in *Pseudomonas putida* OUS82, *J. Bacteriol.*, 176, 2444–2449, 1994.
172. Bosch, R., Garcia-Valdes, E., and Moore, E.R.B., Complete nucleotide sequence and evolutionary significance of a chromosomally encoded naphthalene-degradation lower pathway from *Pseudomonas stutzeri* AN10, *Gene*, 245, 65–74, 2000.
173. Ensley, B.D., Gibson, D.T., and Laborde, A.L., Oxidation of naphthalene by a multicomponent enzyme system from *Pseudomonas* sp. strain NCIB 9816, *J. Bacteriol.*, 149, 948–954, 1982.
174. Irie, S., Doi, S., Yorifuji, T., Takagi, M., and Yano, K., Nucleotide sequencing and characterization of the genes encoding benzene oxidation enzymes of *Pseudomonas putida*, *J. Bacteriol.*, 169, 5174–5179, 1987.
175. Geary, P.J., Mason, J.R., and Joannou, C.L., Benzene dioxygenase from *Pseudomonas putida* ML2 (NCIB 12190), *Methods Enzymol.*, 188, 52–60, 1990.
176. Tan, H.-M., Tang, H.-Y., Joannou, C.L., Abdel-Wahab, N.H., and Mason, J.R., The *Pseudomonas putida* ML2 plasmid-encoded genes for benzene dioxygenase are unusual in codon usage and low in G+C content, *Gene*, 130, 33–39, 1993.
177. Zylstra, G.J. and Gibson, D.T., Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *E. coli*, *J. Biol. Chem.*, 264, 14940–14946, 1989.
178. Mosqueda, G., Ramos-Gonzalez, M.I., and Ramos, J.L., Toluene metabolism by the solvent-tolerant *Pseudomonas putida* DOT-T1 strain, and its role in solvent impermeabilization, *Gene*, 232, 69–76, 1999.
179. Corkery, D.M. and Dobson, A.D., Reverse transcription-PCR analysis of the regulation of ethylbenzene dioxygenase gene expression in *Pseudomonas fluorescens* CA-4, *FEMS Microbiol. Lett.*, 166, 171–176, 1998.
180. Warhurst, A.M., Clarke, K.F., Hill, R.A., Holt, R.A., and Fewson, C.A., Metabolism of styrene by *Rhodococcus rhodochrous* NCIMB 13259, *Appl. Environ. Microbiol.*, 60, 1137–1145, 1994.
181. Junker, L. and Hay, A.G., Preliminary evidence for bacterial growth on styrene via the *tod* pathway, in *Abstracts of the 102nd General Meeting of the American Society for Microbiology 2002*, American Society for Microbiology, 2002.
182. Junker, L. and Hay, A.G., Bacterial growth on styrene using a mosaic pathway, in *Abstracts of the 103rd General Meeting of the American Society for Microbiology 2003*, American Society for Microbiology, 2003.
183. Boyd, D.R., Sharma, N.D., Byrne, B., Hand, M.V., Malone, J.F., Sheldrake, G.N., Blacker, J., and Dalton, H., Enzymatic and chemoenzymatic synthesis and stereochemical assignment of *cis*-dihydrodiol derivatives of monosubstituted benzenes, *J. Chem. Soc. Perkin Trans.*, 1, 1935–1943, 1998.
184. Eaton, R.W. and Timmis, K.N., Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in *Pseudomonas putida* RE204, *J. Bacteriol.*, 168, 123–131, 1986.
185. Aoki, H., Kimura, T., Habe, H., Yamane, H.T.K., and Omori, T., Cloning, nucleotide sequence, and characterization of the genes encoding enzymes involved in the degradation of cumene to 2-hydroxy-6-oxo-7-methylocta-2,4-dienoic acid in *Pseudomonas fluorescens* IP01, *J. Ferment. Bioeng.*, 81, 187–196, 1996.
186. Dabrock, B., Riedel, J., Bertram, J., and Gottschalk, G., Isopropylbenzene (cumene)—a new substrate for the isolation of trichloroethene-degrading bacteria, *Arch. Microbiol.*, 158, 9–13, 1992.
187. Kim, D., Kim, Y.S., Kim, S.K., Kim, S.W., Zylstra, G.J., Kim, Y.M., and Kim, E., Monocyclic aromatic hydrocarbon degradation by *Rhodococcus* sp. strain DK17, *Appl. Environ. Microbiol.*, 68, 3270–3278, 2002.

188. Furukawa, K., Arimura, N., and Miyazaki, T., Nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene of *Pseudomonas pseudoalcaligenes*, *J. Bacteriol.*, 169, 427–429, 1987.
189. Erickson, B.D. and Mondello, F.J., Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated biphenyl-degrading enzyme in *Pseudomonas* strain LB400, *J. Bacteriol.*, 174, 2903–2912, 1992.
190. Sylvestre, M., Sirois, M., Hurtubise, Y., Bergeron, J., Ahmad, D., Shareck, F., Barriault, D., Guillemette, I., and Juteau, J.M., Sequencing of *Comamonas testosteroni* strain B-356-biphenyl/chlorobiphenyl dioxygenase genes: evolutionary relationships among Gram-negative bacterial biphenyl dioxygenases, *Gene*, 174, 195–202, 1996.
191. Masai, E., Yamada, A., Healy, J., Hatta, T., Kimbara, K., Fukuda, M., and Yano, K., Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1, *Appl. Environ. Microbiol.*, 61, 2079–2085, 1995.
192. Asturias, J.A., Díaz, E., and Timmis, K.N., The evolutionary relationship of biphenyl dioxygenase from Gram-positive *Rhodococcus globerulus* P6 to multicomponent dioxygenases from Gram-negative bacteria, *Gene*, 156, 11–18, 1995.
193. Arai, H., Kosono, S., Taguchi, K., Maeda, M., Song, E., Fuji, F., Chung, S.-Y., and Kudo, T., Two sets of biphenyl and PCB degradation genes on a linear plasmid in *Rhodococcus erythropolis* A421, *J. Ferment. Bioeng.*, 86, 595–599, 1998.
194. Hayase, N., Taira, K., and Furukawa, K., *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria, *J. Bacteriol.*, 172, 1160–1164, 1990.
195. Akhtar, M.N., Boyd, D.R., Thompson, N.J., Koreeda, M., Gibson, D.T., Mahadevan, V., and Jerina, D.M., Absolute stereochemistry of the dihydroanthracene-*cis*- and -*trans*-1,2-diols produced from anthracene by mammals and bacteria, *J. Chem. Soc. Perkin Trans.*, 1, 2506–2511, 1975.
196. Jerina, D.M., Selander, H., Yagi, H., Wells, M.C., Davey, J.F., Mahadevan, V., and Gibson, D.T., Dihydrodiols from anthracene and phenanthrene, *J. Am. Chem. Soc.*, 98, 5988–5996, 1976.
197. Saito, A., Iwabuchi, T., and Harayama, S., A novel phenanthrene dioxygenase from *Nocardioideis* sp. strain KP7: expression in *Escherichia coli*, *J. Bacteriol.*, 182, 2134–2141, 2000.
198. Laurie, A.D. and Lloyd-Jones, G., The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism, *J. Bacteriol.*, 181, 531–540, 1999.
199. Krivobok, S., Kuony, S., Meyer, C., Louwagie, M., Willison, J.C., and Jouanneau, Y., Identification of pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1: evidence for two ring-hydroxylating dioxygenases, *J. Bacteriol.*, 185, 3828–3841, 2003.
200. Khan, A.A., Wang, R.F., Cao, W.W., Doerge, D.R., Wennerstrom, D., and Cerniglia, C.E., Molecular cloning, nucleotide sequence, and expression of genes encoding a polycyclic aromatic ring dioxygenase from *Mycobacterium* sp. strain PYR-1, *Appl. Environ. Microbiol.*, 67, 3577–3585, 2001.
201. Walter, U., Beyer, M., Klein, J., and Rehm, H.J., Degradation of pyrene by *Rhodococcus* sp. UW1, *Appl. Microbiol. Biotechnol.*, 34, 671–676, 1991.
202. Story, S.P., Parker, S.H., Hayasaka, S.S., Riley, M.B., and Kline, E.L., Convergent and divergent points in catabolic pathways involved in utilization of fluoranthene, naphthalene, anthracene, and phenanthrene by *Sphingomonas paucimobilis* var. EPA505, *J. Ind. Microbiol. Biotechnol.*, 26, 369–382, 2001.
203. Grifoll, M., Selifonov, S.A., Gatlin, C.V., and Chapman, P.J., Actions of a versatile fluorene-degrading bacterial isolate on polycyclic aromatic compounds, *Appl. Environ. Microbiol.*, 61, 3711–3723, 1995.
204. Selifonov, S.A., Grifoll, M., Gurst, J.E., and Chapman, P.J., Isolation and characterization of (+)-1,1a-dihydroxy-1-hydrofluoren-9-one formed by angular dioxygenation in the bacterial catabolism of fluorene, *Biochem. Biophys. Res. Commun.*, 193, 67–76, 1993.
205. Fujii, T., Takeo, M., and Maeda, Y., Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA, *Microbiology*, 143, 93–99, 1997.
206. Urata, M., Uchida, E., Nojiri, H., Omori, T., Obo, R., Miyaura, N., and Ouchiya, N., Genes involved in aniline degradation by *Delftia acidovorans* strain 7N and its distribution in the natural environment, *Biosci. Biotechnol. Biochem.*, 68, 2457–2465, 2004.

207. Murakami, S., Hayashi, T., Maeda, T., Takenaka, S., and Aoki, K., Cloning and functional analysis of aniline dioxygenase gene cluster, from *Frateuria* species ANA-18, that metabolizes aniline via an *ortho*-cleavage pathway of catechol, *Biosci. Biotechnol. Biochem.*, 67, 2351–2358, 2003.
208. Chang, H.K., Mohseni, P., and Zylstra, G.J., Characterization and regulation of the genes for a novel anthranilate 1,2-dioxygenase from *Burkholderia cepacia* DBO1, *J. Bacteriol.*, 185, 5871–5881, 2003.
209. Neidle, E.L., Hartnett, C., Ornston, L.N., Bairock, A., Rekik, M., and Harayama, S., Nucleotide sequences of the *Acinetobacter calcoaceticus* *benABC* genes for benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases, *J. Bacteriol.*, 173, 5385–5395, 1991.
210. Haddad, S., Eby, D.M., and Neidle, E.L., Cloning and expression of the benzoate dioxygenase genes from *Rhodococcus* sp. strain 19070, *Appl. Environ. Microbiol.*, 67, 2507–2514, 2001.
211. Jeffrey, W.H., Cuskey, S.M., Chapman, P.J., Resnick, S., and Olsen, R.H., Characterization of *Pseudomonas putida* mutants unable to catabolize benzoate: cloning and characterization of *Pseudomonas* genes involved in benzoate catabolism and isolation of a chromosomal DNA fragment able to substitute for *xylS* in activation of the TOL lower-pathway promoter, *J. Bacteriol.*, 174, 4986–4996, 1992.
212. Tsoi, T.V., Plotnikova, E.G., Cole, J.R., Guerin, W.F., Bagdasarian, M., and Tiedje, J.M., Cloning, expression, and nucleotide sequence of the *Pseudomonas aeruginosa* 142 *ohb* genes coding for oxygenolytic *ortho* dehalogenation of halobenzoates, *Appl. Environ. Microbiol.*, 65, 2151–2162, 1999.
213. Suzuki, K., Ogawa, N., and Miyashita, K., Expression of 2-halobenzoate dioxygenase genes (*cbdSABC*) involved in the degradation of benzoate and 2-halobenzoate in *Burkholderia* sp. TH2, *Gene*, 262, 137–145, 2001.
214. Haak, B., Fetzner, S., and Lingens, F., Cloning, nucleotide sequence, and expression of the plasmid-encoded genes for the two-component 2-halobenzoate 1,2-dioxygenase from *Pseudomonas cepacia* 2CBS, *J. Bacteriol.*, 177, 667–675, 1995.
215. Francisco, P.J., Ogawa, N., Suzuki, K., and Miyashita, K., The chlorobenzoate dioxygenase genes of *Burkholderia* sp. strain NK8 involved in the catabolism of chlorobenzoates, *Microbiology*, 147, 121–133, 2001.
216. Nakatsu, C.H., Straus, N.A., and Wyndham, R.C., The nucleotide sequence of the Tn5271 3-chlorobenzoate 3,4-dioxygenase genes (*cbaAB*) unites the class IA oxygenases in a single lineage, *Microbiology*, 141, 485–495, 1995.
217. Eaton, R.W., *p*-Cumate catabolic pathway in *Pseudomonas putida* F1: cloning and characterization of DNA carrying the *cmt* operon, *J. Bacteriol.*, 178, 1351–1362, 1996.
218. Díaz, E., Ferrández, A., and García, J.L., Characterization of the *hca* cluster encoding the dioxygenolytic pathway for initial catabolism of 3-phenylpropionic acid in *Escherichia coli* K-12, *J. Bacteriol.*, 180, 2915–2923, 1998.
219. Hudlicky, T., Stabile, M.R., Gibson, D.T., and Whited, G.M., 1-Chloro-(2*S*, 3*S*)-dihydroxycyclohexa-4,6-diene, *Organic Synth.*, 76, 77–85, 1999.
220. van der Meer, J.R., Van Neerven, A.R.W., De Vries, E.J., De Vos, W.M., and Zehnder, A.J.B., Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51, *J. Bacteriol.*, 173, 6–15, 1991.
221. Beil, S., Happe, B., Timmis, K.N., and Pieper, D.H., Genetic and biochemical characterization of the broad spectrum chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12—dechlorination of 1,2,4,5-tetrachlorobenzene, *Eur. J. Biochem.*, 247, 190–199, 1997.
222. Boyd, D.R., Sharma, N.D., Barr, S.A., Dalton, K., Chima, J., Whited, G., and Seemayer, R., Chemoenzymatic synthesis of the 2,3- and 3,4-*cis*-dihydrodiol enantiomers of monosubstituted benzenes, *J. Am. Chem. Soc.*, 116, 1147–1148, 1994.
223. Seto, M., Kimbara, K., Shimura, M., Hatta, T., Fukuda, M., and Yano, K., A novel transformation of polychlorinated biphenyls by *Rhodococcus* sp. strain RHA1, *Appl. Environ. Microbiol.*, 61, 3353–3358, 1995.

224. Barriault, D., Pelletier, C., Hurtubise, Y., and Sylvestre, M., Substrate selectivity pattern of *Comamonas testosteroni* strain B-356 towards dichlorobiphenyls, *Int. Biodeterior. Biodegrad.*, 39, 311–316, 1997.
225. Gibson, D.T., Cruden, D.L., Haddock, J.D., Zylstra, G.J., and Brand, J.M., Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707, *J. Bacteriol.*, 175, 4561–4564, 1993.
226. Ohtsubo, Y., Shimura, M., Delawary, M., Kimbara, K., Takagi, M., Kudo, T., Ohta, A., and Nagata, Y., A novel approach to the improvement of biphenyl and polychlorinated biphenyl degradation activity: promoter implantation by homologous recombination, *Appl. Environ. Microbiol.*, 69, 146–153, 2003.
227. Bedard, D.L. and Haberl, M.L., Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains, *Microb. Ecol.*, 20, 87–102, 1990.
228. Habe, H., Miyakoshi, M., Chung, J., Kasuga, K., Yoshida, T., Nojiri, H., and Omori, T., Phthalate catabolic gene cluster is linked to the angular dioxygenase gene in *Terrabacter* sp. strain DBF63, *Appl. Microbiol. Biotechnol.*, 61, 44–54, 2003.
229. Ouchiya, N., Miyachi, S., and Omori, T., Cloning and nucleotide sequence of carbazole catabolic genes from *Pseudomonas stutzeri* strain OM1, isolated from activated sludge, *J. Gen. Appl. Microbiol.*, 44, 57–63, 1998.
230. Nam, J.-W., Nojiri, H., Noguchi, H., Uchimura, H., Yoshida, T., Habe, H., Yamane, H., and Omori, T., Purification and characterization of carbazole 1,9a-dioxygenase, a three component dioxygenase system of *Pseudomonas resinovorans* strain CA10, *Appl. Environ. Microbiol.*, 68, 5882–5890, 2002.
231. Chang, H.-K. and Zylstra, G.J., Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1, *J. Bacteriol.*, 180, 6529–6537, 1998.
232. Eaton, R.W., Plasmid-encoded phthalate catabolic pathway in *Arthrobacter keyseri* 12B, *J. Bacteriol.*, 183, 3689–3703, 2001.
233. Wang, Y.Z., Zhou, Y., and Zylstra, G.J., Molecular analysis of isophthalate and terephthalate degradation by *Comamonas testosteroni* YZW-D, *Environ. Health Perspect.*, 103(Suppl. 5), 9–12, 1995.
234. Schl fli, H.R., Weiss, M.A., Leisinger, T., and Cook, A.M., Terephthalate 1,2-dioxygenase system from *Comamonas testosteroni* T-2: purification and some properties of the oxygenase component, *J. Bacteriol.*, 176, 6644–6652, 1994.
235. Shigematsu, T., Yumihara, K., Ueda, Y., Morimura, S., and Kida, K., Purification and gene cloning of the oxygenase component of the terephthalate 1,2-dioxygenase system from *Delftia tsuruhatensis* strain T7, *FEMS Microbiol. Lett.*, 220, 255–260, 2003.
236. Harayama, S., Rekik, M., Bairoch, A., Neidle, E.L., and Ornston, L.N., Potential DNA slippage structure acquired during evolutionary divergence of *Acinetobacter calcoaceticus* chromosomal *benABC* and *Pseudomonas putida* TOL pWWO plasmid *xylXYZ*, genes encoding benzoate dioxygenases, *J. Bacteriol.*, 173, 7540–7548, 1991.
237. Bernhardt, F.-H., Bill, E., Trautwein, A.X., and Twilfer, H., 4-Methoxybenzoate monooxygenase from *Pseudomonas putida*: isolation, biochemical properties, substrate specificity, and reaction mechanisms of the enzyme components, *Methods Enzymol.*, 161, 281–294, 1988.
238. Locher, H.H., Leisinger, T., and Cook, A.M., 4-Toluene sulfonate methyl-monooxygenase from *Comamonas testosteroni* T-2: purification and some properties of the oxygenase component, *J. Bacteriol.*, 173, 3741–3748, 1991.
239. Mampel, J., Ruff, J., Junker, F., and Cook, A.M., The oxygenase component of the 2-aminobenzenesulfonate dioxygenase system from *Alcaligenes* sp. strain O-1, *Microbiology*, 145, 3255–3264, 1999.
240. Lessner, D.J., Johnson, G.R., Parales, R.E., Spain, J.C., and Gibson, D.T., Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765, *Appl. Environ. Microbiol.*, 68, 634–641, 2002.
241. Robertson, J.B., Spain, J.C., Haddock, J.D., and Gibson, D.T., Oxidation of nitrotoluene by toluene dioxygenase: evidence for a monooxygenase reaction, *Appl. Environ. Microbiol.*, 58, 2643–2648, 1992.

242. Parales, J.V., Kumar, A., Parales, R.E., and Gibson, D.T., Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42, *Gene*, 181, 57–61, 1996.
243. Parales, R.E., Emig, M.D., Lynch, N.A., and Gibson, D.T., Substrate specificities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems, *J. Bacteriol.*, 180, 2337–2344, 1998.
244. Suen, W.-C. and Spain, J.C., Cloning and characterization of *Pseudomonas* sp. strain DNT genes for 2,4-dinitrotoluene degradation, *J. Bacteriol.*, 175, 1831–1837, 1993.
245. Nishino, S.F., Paoli, G.C., and Spain, J.C., Aerobic degradation of dinitrotoluenes and the pathway for bacterial degradation of 2,6-dinitrotoluene, *Appl. Environ. Microbiol.*, 66, 2139–2147, 2000.
246. Seeger, M., Camara, B., and Hofer, B., Dehalogenation, denitration, dehydrogenation, and angular attack on substituted biphenyls and related compounds by biphenyl dioxygenase, *J. Bacteriol.*, 183, 3548–3555, 2001.
247. Bestetti, G., Bianchi, D., Bosetti, A., Di Gennaro, P., Galli, E., Leoni, B., Pelizzoni, F., and Sello, G., Bioconversion of substituted naphthalenes to the corresponding 1,2-dihydro-1,2-dihydroxy derivatives. Determination of the regio- and stereochemistry of the oxidation reactions, *Appl. Microbiol. Biotechnol.*, 44, 306–313, 1995.
248. Deluca, M.E. and Hudlicky, T., Microbial oxidation of naphthalene derivatives. Absolute configuration of metabolites, *Tetrahedron Lett.*, 31, 13–16, 1990.
249. Whited, G.M., Downie, J.C., Hudlicky, T., Fearnley, S.P., Dudding, T.C., Olivo, H.F., and Parker, D., Oxidation of 2-methoxynaphthalene by toluene, naphthalene and biphenyl dioxygenases: structure and absolute stereochemistry of metabolites, *Bioorg. Med. Chem.*, 2, 727–734, 1994.
250. Brilon, C., Beckmann, W., and Knackmuss, H.-J., Catabolism of naphthalenesulfonic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22, *Appl. Environ. Microbiol.*, 42, 44–55, 1981.
251. Knackmuss, H.-J., Beckmann, W., and Otting, W., Microbiological synthesis of (+)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene-2-carboxylic acid, *Angew. Chem. Int. Ed. Engl.*, 15, 549, 1976.
252. Martin, V.J.J. and Mohn, W.W., A novel aromatic-ring-hydroxylating dioxygenase from the diterpenoid-degrading bacterium *Pseudomonas abietaniphila* BKME-9, *J. Bacteriol.*, 181, 2675–2682, 1999.
253. Habe, H., Ashikawa, Y., Saiki, Y., Yoshida, T., Nojiri, H., and Omori, T., *Sphingomonas* sp. strain KA1, carrying a carbazole dioxygenase gene homologue, degrades chlorinated dibenzo-*p*-dioxins in soil, *FEMS Microbiol. Lett.*, 211, 43–49, 2002.
254. Shepherd, J.M. and Lloyd-Jones, G., Novel carbazole degradation genes of *Sphingomonas* CB3: sequence analysis, transcription, and molecular ecology, *Biochem. Biophys. Res. Commun.*, 247, 129–135, 1998.
255. Resnick, S.M., Torok, D.S., and Gibson, D.T., Oxidation of carbazole to 3-hydroxycarbazole by naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase, *FEMS Microbiol. Lett.*, 113, 297–302, 1993.
256. Iida, T., Mukouzaka, Y., Nakamura, K., and Kudo, T., Plasmid-borne genes code for an angular dioxygenase involved in dibenzofuran degradation by *Terrabacter* sp. strain YK3, *Appl. Environ. Microbiol.*, 68, 3716–3723, 2002.
257. Kasuga, K., Habe, H., Chung, J.S., Yoshida, T., Nojiri, H., Yamane, H., and Omori, T., Isolation and characterization of the genes encoding a novel oxygenase component of angular dioxygenase from the gram-positive dibenzofuran-degrader *Terrabacter* sp. strain DBF63, *Biochem. Biophys. Res. Commun.*, 283, 195–204, 2001.
258. Takagi, T., Habe, H., Yoshida, T., Yamane, H., Omori, T., and Nojiri, H., Characterization of [3Fe-4S] ferredoxin DbfA3, which functions in the angular dioxygenase system of *Terrabacter* sp. strain DBF63, *Appl. Microbiol. Biotechnol.*, 68, 336–345, 2005.
259. Armengaud, J., Gaillard, J., and Timmis, K.N., A second [2Fe-2S] ferredoxin from *Sphingomonas* sp. strain RW1 can function as an electron donor for the dioxin dioxygenase, *J. Bacteriol.*, 182, 2238–2244, 2000.
260. Armengaud, J., Happe, B., and Timmis, K.N., Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome, *J. Bacteriol.*, 180, 3954–3966, 1998.

261. Armengaud, J. and Timmis, K.N., Molecular characterization of Fdx1, a putidaredoxin-type [2Fe-2S] ferredoxin able to transfer electrons to the dioxin dioxygenase of *Sphingomonas* sp. RW1, *Eur. J. Biochem.*, 247, 833–842, 1997.
262. Armengaud, J. and Timmis, K.N., The reductase RedA2 of the multi-component dioxin dioxygenase system of *Sphingomonas* sp. RW1 is related to class-I cytochrome P450-type reductases, *Eur. J. Biochem.*, 253, 437–444, 1998.
263. Rosche, B., Tshisuaka, B., Hauer, B., Lingens, F., and Fetzner, S., 2-Oxo-1,2-dihydroquinoline 8-monooxygenase: phylogenetic relationships to other multicomponent nonheme iron oxygenases, *J. Bacteriol.*, 179, 3549–3554, 1997.
264. Sauber, K., Fröhner, C., Rosenberg, G., Eberspächer, J., and Lingens, F., Purification and properties of pyrazon dioxygenase from pyrazon-degrading bacteria, *Eur. J. Biochem.*, 74, 89–97, 1977.
265. Treadway, S.L., Yanagimachi, K.S., Lankenau, E., Lessard, P.A., Stephanopoulos, G., and Sinskey, A.J., Isolation and characterization of indene bioconversion genes from *Rhodococcus* strain I24, *Appl. Microbiol. Biotechnol.*, 51, 786–793, 1999.
266. Eaton, S.E., Resnick, S.M., and Gibson, D.T., Initial reactions in the oxidation of 1,2-dihydronaphthalene metabolism by *Sphingomonas yanoikuyae* strains, *Appl. Environ. Microbiol.*, 62, 4388–4394, 1996.
267. Sikkema, J. and De Bont, J.A.M., Metabolism of tetralin (1,2,3,4-tetrahydronaphthalene) in *Corynebacterium* sp. strain C125, *Appl. Environ. Microbiol.*, 59, 567–572, 1993.
268. Moreno-Ruiz, E., Hernaez, M.J., Martinez-Perez, O., and Santero, E., Identification and functional characterization of *Sphingomonas macrogolitabida* strain TFA genes involved in the first two steps of the tetralin catabolic pathway, *J. Bacteriol.*, 185, 2026–2030, 2003.
269. Dehmel, U., Engesser, K.-H., Timmis, K.N., and Dwyer, D.F., Cloning, nucleotide sequence, and expression of the gene encoding a novel dioxygenase involved in metabolism of carboxydiphenyl ethers in *Pseudomonas pseudoalcaligenes* POB310, *Arch. Microbiol.*, 163, 35–41, 1995.
270. Boyd, D.R., Sharma, N.D., and Allen, C.C.R., Aromatic dioxygenases: molecular biocatalysis and applications, *Curr. Opin. Biotechnol.*, 12, 564–573, 2001.

11 A Genomic Approach to Investigating Baker's Yeast Reductions

Jon D. Stewart

CONTENTS

11.1	Introduction to Yeast-Mediated Reductions	334
11.1.1	Evidence for Multiple Yeast Reductases.....	334
11.1.2	Improving the Stereoselectivities of Yeast-Mediated Reductions	335
11.1.3	Defining the Full Complement of <i>Saccharomyces cerevisiae</i> Reductases.....	337
11.2	A Library of Individual Yeast Reductases.....	337
11.2.1	Creating the Library	337
11.2.2	Applications of Yeast GST-Fusion Proteins.....	341
11.2.2.1	Ethyl 4-Chloroacetoacetate	342
11.2.2.2	Ethyl 4-Phenyl-2-Oxobutyrate.....	343
11.2.2.3	Atorvastatin Building Block.....	343
11.2.2.4	Taxol Side-Chain Precursor	344
11.2.3	Large-Scale Reductions.....	345
11.3	Summary and Future Outlook.....	346
11.3.1	Judging Completeness in a Collection.....	347
11.3.2	Expanding the Dehydrogenase Collection	347
11.3.3	Wider Applicability and Limitations	348
	Acknowledgments	348
	References	349

Alcohols are one of the most common functional groups in organic chemistry, either as constituents of the final products or as “handles” for further synthetic transformations. While all primary alcohols are achiral, this is not necessarily true of secondary and tertiary alcohols. For these cases, controlling the absolute stereochemistry is always essential in modern synthesis. Solutions to this problem include kinetic resolutions by acylation or deacylation, stereoselective oxidations, and asymmetric reductions [1–3]. The last method—asymmetric ketone reduction—is particularly powerful as it potentially allows 100% of the starting material to be transformed into the desired enantiomer, as compared with kinetic resolution strategies that have maximal 50% yields.

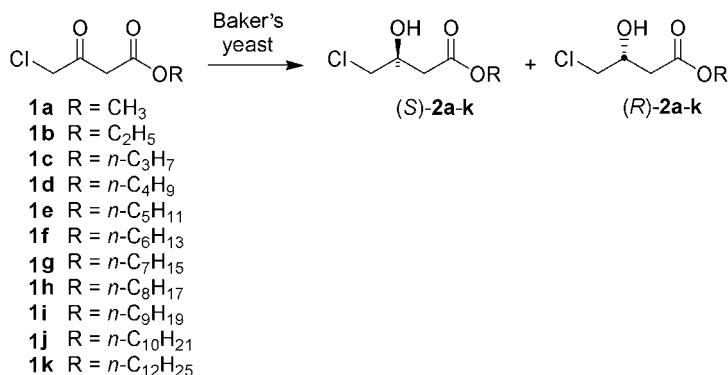
11.1 INTRODUCTION TO YEAST-MEDIATED REDUCTIONS

Among the many biocatalytic approaches to asymmetric ketone reductions that have been explored [4], the use of commercial baker's yeast has been by far the most popular one [5,6]. Whole cells of this organism are readily available, and inexpensive, from the brewing and baking industries. The experimental protocols are very simple: dried yeast cells are rehydrated in the presence of a carbohydrate (usually sucrose), the substrate is added and allowed to react, then the alcohol product is recovered by solvent extraction [7]. Yeast-mediated reductions are also applicable to a very wide range of aldehydes and ketone structures. In fact, it is difficult to find a substrate that is not reduced by baker's yeast.

Unfortunately, several problems counterbalance the many advantages of baker's yeast. Most critically, reductions using whole yeast cells often afford stereoisomeric mixtures of alcohols. In principle, this could be the result of either a single reductase with limited enantioselectivity or multiple enzymes with conflicting stereopreferences. In fact, the latter situation prevails in baker's yeast. This understanding has been the basis of most approaches to improve the stereoselectivities of yeast-mediated reductions, while still maintaining experimental simplicity. Before yeast reductions can be improved rationally, however, the number and identity of the participating enzymes must be established. Even early suggestions that less than half-a-dozen enzymes played key roles turned out to be incorrect, and it is now recognized that several dozens of yeast reductases may be involved. Because the efforts leading to this conclusion have had a direct impact on the development of our genome mining strategy (described below), these results are summarized briefly below.

11.1.1 EVIDENCE FOR MULTIPLE YEAST REDUCTASES

Alcohols with the general structure (*R*)-**2** serve as precursors for pharmaceuticals and nutritional supplement L-carnitine (Scheme 11.1). Because it is commercially available, ethyl ester **1b** is the most convenient substrate; unfortunately, employing baker's yeast under standard conditions affords a 55% ee favoring the (*S*)-enantiomer [8]. Sih and coworkers explored a number of approaches to improving the stereoselectivity of this process, and their results revealed a great deal about the biochemistry of yeast-mediated reductions. Most importantly, they showed that enantioselectivity varied with the concentration of **1b**. If a single reductase were responsible for the conversion, the stereoselectivity would be concentration-independent; however, if two or more enzymes with different stereopreferences accepted **1b**, then decreasing the substrate concentration would favor reduction by the enzyme(s) with the lower K_M value(s). Sih's study provided unequivocal evidence that more than one yeast enzyme was involved in reducing exogenous ketones.



SCHEME 11.1

To define the number and the properties of yeast enzymes that reduced 4-chloroacetoacetate esters, independent groups led by Sih and Nakamura purified yeast protein extracts on standard chromatographic supports [9,10]. When fractions were assayed for the ability to reduce **1b**, four peaks of enzyme activity were obtained, and each was purified to apparent homogeneity. Significantly, each purified protein reduced **1b** with >98% ee, although two were (*R*)-selective and two (*S*)-selective. The simultaneous participation of these four reductases neatly explained the modest enantioselectivities observed for reactions involving whole yeast cells. At least for β -keto ester substrates such as **1b**, it was believed that this collection of four yeast reductases was complete. Later work showed that this was not the case, however.

11.1.2 IMPROVING THE STEREOSELECTIVITIES OF YEAST-MEDIATED REDUCTIONS

Knowing the properties of the four purified yeast reductases opened the door to improving the stereoselectivities of whole-cell reductions by selectively depressing the catalytic activities of specific enzymes. One approach—already mentioned above—is to use low substrate concentrations, which encourages reduction by the enzyme(s) that binds the ketone most tightly. In the case of **1b**, however, incomplete stereoselectivity was still observed even at the lowest practical substrate concentration [8]. Moreover, this approach yields dilute product streams and diminished volumetric productivities that are undesirable in large-scale operations. Altering the substrate structure to restrict acceptance to a limited subset of reductases proved to be a more fruitful approach. Yeast-mediated reductions of a homologous series of 4-chloroacetoacetates differing in their alkoxy moieties revealed that essentially complete (*R*)-selectivity was obtained for side chains longer than C₆ (Figure 11.1) [8]. While such a strategy was successful in this particular case, it is not always possible (or desirable) to modify the substrate structure. This limits the applicability of the approach.

Eliminating the catalytic activities of one or more competing enzymes with undesirable stereoselectivities is a more direct approach to improving yeast-mediated reductions, and several methods for achieving this goal have been reported. Studies of the four purified yeast

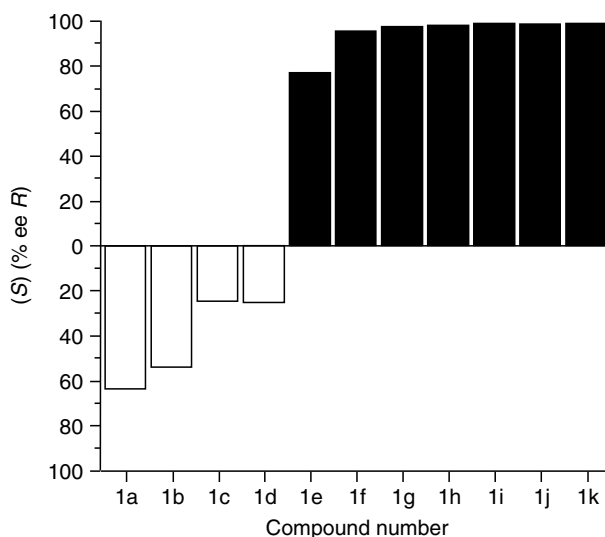


FIGURE 11.1 Yeast-mediated reductions of alkyl 4-chloroacetoacetates. Compound structures are depicted in [Scheme 11.1](#). White bars indicate reactions that proceed with predominant (*S*)-selectivity, while those with predominant (*R*)-selectivity are represented by black bars.

reductases accepting **1b** revealed that reagents such as allyl alcohol (an acrolein precursor), methyl vinyl ketone, and ethyl chloroacetate inactivated particular yeast enzymes [10]. *In vitro*, these were highly selective inactivators, and they could also be used *in vivo* (Figure 11.2). In favorable cases, these additives afforded very high stereoselectivities in whole cell-mediated reductions. However, because the additives are reactive compounds, they are also toxic toward the cells and this always requires the increasing cell mass:substrate to compensate. The increased biomass and the presence of additives complicate downstream processing and purification. Despite these drawbacks, the inclusion of reductase poisons has great practical utility and remains the most common strategy for improving stereoselectivities in yeast reductions [11]. In the case of ethyl 4-chloroacetoacetate **1b**, the (*S*)-alcohol was available in 90% ee and the (*R*)-enantiomer in 80% ee by using methyl vinyl ketone and ethyl chloroacetate, respectively (Figure 11.2).

Knocking out the genes encoding competing reductases provides an even more direct route to eliminating unwanted enzymatic activities. Knowing that the fatty acid synthase complex was one of the D-selective reductases for **1b**, Sih substituted whole cells of a fatty acid synthase mutant strain [12,13] and observed improved stereoselectivity. We extended this strategy to include the other two L-specific reductases isolated by Sih and Nakamura (products of the *YPR1* and *GRE2* genes), and also added gene overexpression to improve stereoselectivity even further. We created a complete set of modified yeast strains in which one of these three proteins was overexpressed, while the other two were ablated by gene knockout [14,15]. The resulting strains were screened for the reduction of a panel of β -keto esters. Knocking out fatty acid synthase virtually eliminated the formation of D-alcohols, and its overexpression enhanced their production, in line with our expectations. By contrast, more complex results were obtained from strains with altered levels of proteins encoded by the *YPR1* and the *GRE2* genes. While their overexpression slightly increased the levels of the corresponding alcohol products, knocking these genes out had almost no effect on the

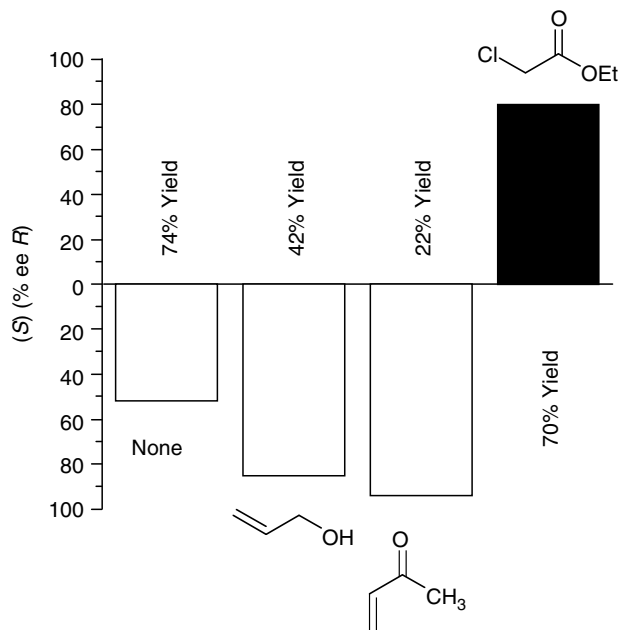


FIGURE 11.2 Yeast-mediated reductions of ethyl 4-chloroacetoacetate in the presence of additives. Inhibitor structures and the corresponding stereoselectivities are indicated.

reduction stereoselectivities. These observations provided the first clues that the baker's yeast genome actually encoded more than three reductases that accepted exogenous β -keto ester substrates such as **1b**.

11.1.3 DEFINING THE FULL COMPLEMENT OF *SACCHAROMYCES CEREVISIAE* REDUCTASES

The surprising results from reductase gene knockout studies made it clear that the complete set of enzymes accepting exogenous ketones had to be identified before we could take full advantage of the biocatalytic opportunities offered by baker's yeast. As previous biochemical approaches had yielded only a subset of the participating enzymes, we chose a bioinformatics approach to answering the question, "How many reductases can potentially be produced by *S. cerevisiae*?" We analyzed the complete genome sequence using a variety of criteria and probe sequences drawn from all major classes of reductases (lactate dehydrogenases, alcohol dehydrogenases, aldose reductases, D-hydroxyacid dehydrogenases, short- and medium-chain dehydrogenases) and discovered that approximately 50 genes likely encode reductases (Table 11.1) [16]. While the properties of a few of these—the well-known yeast alcohol dehydrogenases and the gene products of *YPRI* and *GRE2*, for example—had been delineated prior to our work, the rest were almost completely unknown.

Given the large number of reductases that could be expressed in a yeast cell, it was clear that attempting to create mutant strains with dozens of genes knocked out simultaneously was not feasible. It also seemed difficult to find inhibitors that would be sufficiently selective to eliminate the catalytic activities of only a few of the 50 potential reductases. Rather than continuing to use whole baker's yeast cells for asymmetric reductions, we decided instead to focus on the individual reductases. In this approach, yeast cells are treated as a genetic resource rather than a self-contained biocatalytic reagent. In addition to solving practical problems in asymmetric ketone reductions, we were also drawn to this strategy because it might be generally useful. Our methods can be applied to any genome or chemical conversion. All that is required is one or more amino acid sequences from proteins known to carry out the reaction of interest and the readily available computer software.

There were two key problems in moving from whole baker's yeast cells to the use of individual yeast reductases. First, each purified protein must be available, preferably by simple standard method that can be applied to large numbers of enzymes in parallel. Second, methods for carrying out reactions on preparative scales must be available. Our approaches to solving these two problems are described below.

11.2 A LIBRARY OF INDIVIDUAL YEAST REDUCTASES

11.2.1 CREATING THE LIBRARY

At the start of our work, almost no yeast reductases were available commercially in purified form. We, therefore, turned to a recombinant collection developed by Martzen and coworkers in which every open reading frame (ORF) from the baker's yeast genome was overexpressed in individual yeast strains (Figure 11.3) [17]. Each protein was fused to a common tag (glutathione *S*-transferase) that allowed them to be purified by a common, one-step method using a glutathione-containing solid support. The purified fusion proteins could then be assayed for catalytic activity by conventional methods. This collection is ideally suited to analyzing the substrate- and stereoselectivities of yeast reductases rapidly. A given ketone can be tested as a substrate for each protein in small-scale reactions. This defines the subset of yeast reductases that accept the substrate and the stereoselectivities of the reactions, and allows an informed choice of the best reductase for large-scale bioconversions.

TABLE 11.1***Saccharomyces cerevisiae* Genes Encoding Potential Ketone Reductases**

Protein	Gene(s)	ORF(s)	Plasmid
Lactate dehydrogenases			
L-Lactate cytochrome <i>c</i> oxidoreductase	<i>CYB2</i>	Yml054c	
D-Lactate dehydrogenase	<i>DLD1</i>	Ydl174c	
D-Lactate dehydrogenase	<i>DLD2</i>	Ydl178w	
D-Lactate dehydrogenase	<i>DLD3</i>	Yel071w	
Alcohol dehydrogenases			
Long-chain alcohol dehydrogenase	<i>SFA1</i>	Ydl168w	
Alcohol dehydrogenase I	<i>ADH1</i>	Yol086c	
Alcohol dehydrogenase II	<i>ADH2</i>	Ymr303c	
Alcohol dehydrogenase III	<i>ADH3</i>	Ymr083w	
Alcohol dehydrogenase V	<i>ADH5</i>	Ybr145w	
Alcohol dehydrogenase IV	<i>ADH4</i>	Ygl256w	
Medium-chain alcohol dehydrogenases			
Sorbitol dehydrogenase	<i>SOR1</i>	Yjr159w	pIK31
Xylitol dehydrogenase	<i>XYL2</i>	Ylr070c	pIK23
Putative polyol dehydrogenase	—	Yal061w	pIK28
(2 <i>R</i> ,3 <i>R</i>)-2,3-Butanediol dehydrogenase	<i>BDH1</i>	Yal060w	pTM3
NADPH-dependent dehydrogenase	<i>ADH6</i>	Ymr318c	pIK25
NADPH-dependent dehydrogenase	<i>ADH7</i>	Ycr105w	pIK32
ζ-Crystallin homolog	<i>ZTA1</i>	Ybr046c	
Short-chain alcohol dehydrogenases			
NADPH-dependent methyl glyoxal reductase	<i>GRE2</i>	Yol151w	pIK3
Putative oxidoreductase	—	Ydr541c	pIK5
Oxidoreductase	—	Ygl157w	pIK7
Oxidoreductase	—	Ygl039w	pIK6
Microsomal β-keto reductase	—	Ylr426w	
Putative oxidoreductase	—	Ybr159w	
Putative oxidoreductase	—	Ydl114w	
NADP ⁺ -dependent dehydrogenase	—	Ymr226c	
Putative oxidoreductase	—	Yir035c	
Putative oxidoreductase	—	Yir036c	
NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase	<i>AYR1</i>	Yil124w	
Putative oxidoreductase	—	Ykl107w	
3-Oxoacyl-(acyl-carrier-protein) reductase	<i>OAR1</i>	Ykl055c	
Putative aryl alcohol dehydrogenase	<i>AAD14</i>	Ynl331c	pIK11
Putative aryl alcohol dehydrogenase	<i>AAD3</i>	Ycr107w	pIK10
Putative aryl alcohol dehydrogenase	<i>AAD4</i>	Ydl243c	
Putative aryl alcohol dehydrogenase	<i>AAD10</i>	Yjr155w	
Putative aryl alcohol dehydrogenase	<i>AAD16</i>	Yfl057c	
Putative aryl alcohol dehydrogenase	<i>AAD15</i>	Yol165c	
Aldose reductase family			
2-Methylbutyraldehyde reductase	<i>YPR1</i>	Ydr368w	pIK4
Putative NADP ⁺ coupled glycerol dehydrogenase	<i>GCY1</i>	Yor120w	pIK30
Large subunit of NADP ⁺ -dependent arabinose dehydrogenase	<i>ARA1</i>	Ybr149w	pIK12
Aldose reductase	<i>GRE3</i>	Yhr104w	pIK29
Putative oxidoreductase	—	Yjr096w	pIK9
NADPH-dependent α-keto amide reductase	—	Ydl124w	pIK8

TABLE 11.1 (continued)

***Saccharomyces cerevisiae* Genes Encoding Potential Ketone Reductases**

Protein	Gene(s)	ORF(s)	Plasmid
D-Hydroxyacid dehydrogenase family			
Putative dehydrogenase	—	Ypl113c	pIK15
Putative hydroxyacid dehydrogenase	—	Ygl185c	pAKS1
Putative hydroxyisocaproate dehydrogenase	—	Ynl274c	pIK13
NAD ⁺ -dependent formate dehydrogenase	<i>FDH1</i>	Yor388c	pIK14
NAD ⁺ -dependent formate dehydrogenase	<i>FDH2</i>	Ypl275w	pIK18
Multifunctional			
Fatty acid synthase	<i>FAS1</i> , <i>FAS2</i>	Ykl182w, Ypl231w	

The original Martzen library encompasses all 6144 ORFs in the yeast genome, and it was designed to allow the enzyme(s) catalyzing specific reactions to be identified directly, with no additional information required. We deliberately restricted ourselves to a subset of this library, since our prior genome analysis had focused attention on approximately 50 ORFs most likely to participate in ketone reductions. We narrowed this list further by eliminating proteins known to have narrow substrate specificities, for example lactate and alcohol dehydrogenases, and those with weak sequence similarity to authentic reductases. This left 23 proteins for our first-pass library (Table 11.1). Clearly, the number can be increased in the future, by including additional proteins either from the *S. cerevisiae* genome or from those of other organisms.

Applying yeast reductase GST-fusions to nonnatural substrates and millimolar substrate concentrations required relatively higher protein concentrations in the screening reactions. Originally, the GST-fusion proteins were overproduced in *S. cerevisiae*. While this provided

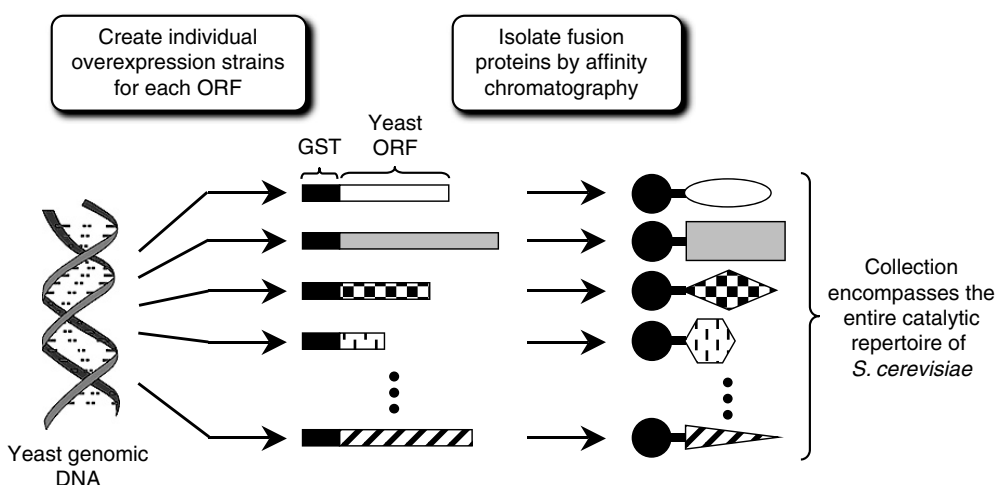


FIGURE 11.3 Overall strategy for creating a GST-fusion protein library from the yeast genome. DNA encoding the GST portion is shown in black and yeast ORFs (and their protein products) are indicated by different patterns.

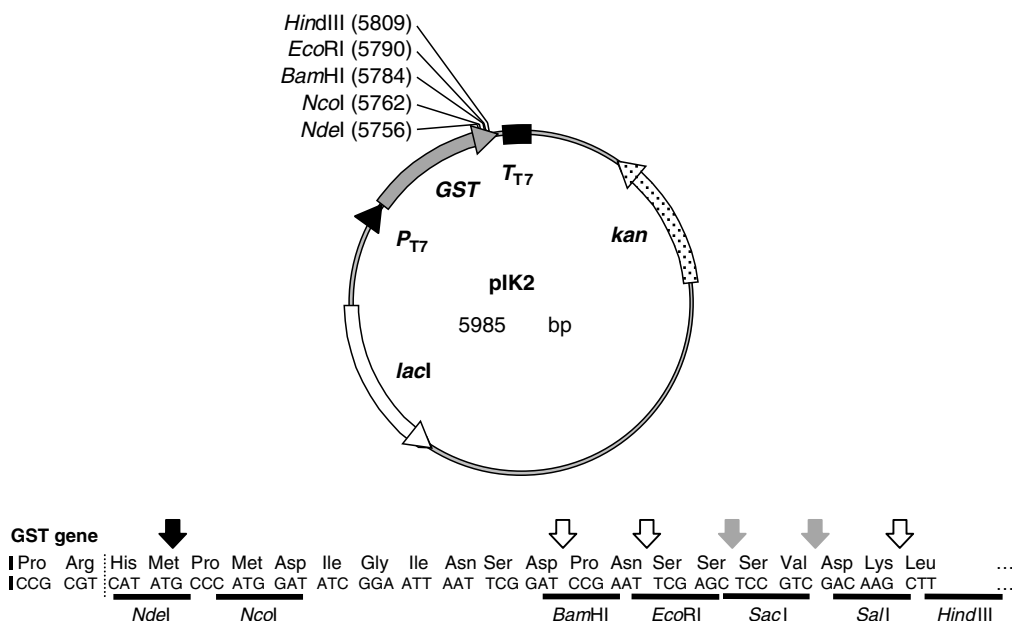


FIGURE 11.4 Structure of plasmid pIK2 is used to overproduce GST-fusion proteins. The locations of key restriction sites are indicated along with important genetic elements. The DNA at the 3'-end of the GST portion is shown below. Arrows indicate the locations of restriction sites, those shown in grey are not unique.

the most “natural” environment for yeast proteins, it also severely limited the quantities that could be obtained. We therefore expressed these the proteins in *Escherichia coli*. Plasmid pIK2 was developed for this purpose, and it includes the strong T7 promoter to direct overproduction of the mRNA corresponding to the GST-fusion protein (Figure 11.4). Two restriction sites (*NdeI* and *NcoI*) are provided for linking the 5'-end of the yeast ORF to the 3'-end of the glutathione *S*-transferase gene as well as several downstream restriction sites for incorporating the yeast ORF. After construction, each plasmid was used to transform *E. coli* strain BL21(DE3). Detailed experimental protocols for isolating and employing these fusion proteins are given below:

1. Isolation of GST-fusion proteins

A culture of the appropriate overexpression strain grown overnight in LB medium supplemented with 25 µg/mL kanamycin was diluted 1:100 into 500 mL of the same medium in a 2 L baffled flask. The culture was shaken at 37°C until the O.D.₆₀₀ was between 0.5 and 1.0, isopropylthio-β-D-galactoside was added to final concentration of 100 µM and the culture was shaken for an additional 6 h at room temperature. Cells harvested by centrifugation were washed twice with cold water, then resuspended in 25 mL of 100 mM KPi, pH 7.0. A cold French pressure cell was used to lyse the cells, then the extract was clarified by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant was mixed with an equal volume of cold 50 mM Tris-Cl, 4 mM MgCl₂, 1 mM DTT, 10% glycerol, pH 7.5 and loaded onto a 2.4 × 5.0 cm column of glutathione resin (Clontech) at a flow rate of 0.5 mL/min. The column had been equilibrated with 50 mM Tris-Cl, 4 mM MgCl₂, 1 mM DTT, 500 mM NaCl, 10% glycerol, pH 7.5 (wash buffer). The flow-through was discarded and the resin was washed twice with 20 mL of the starting buffer. This material was also discarded. Essentially pure

GST-fusion proteins were eluted with 40 mL of freshly prepared elution buffer (starting buffer (39.6 mL) plus 2 M NaOH (0.40 mL), and solid glutathione (0.31 g)). The eluant was dialyzed against several changes of 20 mM Tris-Cl, 2 mM EDTA, 4 mM MgCl₂, 1 mM DTT, 55 mM NaCl, 50% glycerol, pH 7.5 prior to storage at -20°C. The glutathione agarose resin was regenerated by washing with 20 column volumes of phosphate-buffered saline supplemented with 3 M NaCl (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 3.14 M NaCl, pH 7.4) followed by 10 column volumes of wash buffer. We have used a single column for purifying all GST-fusion proteins and have observed no cross-contamination problems.

2. Small-scale reactions involving GST-fusion proteins

NADP⁺ (0.20 μmol, 0.15 mg), glucose-6-phosphate (14 μmol, 4.3 mg), glucose-6-phosphate dehydrogenase (5 μg), and ketone substrate (5 mM) were mixed with the appropriate purified GST-fusion protein (5–50 μg) in 1.0 mL of 100 mM KP_i, pH 7.0. Reaction mixtures were incubated at 30°C and periodically sampled for analysis by normal- and chiral-phase GC (DB-17 (J&W Scientific) and Chirasil-Dex CB (Varian) or Chirasil-Val (Varian) columns, respectively). Reactions were scaled up by 10- or 20-fold to allow product isolation and spectroscopic characterization. At the conclusion of the bioconversion, the reaction mixture was extracted with Et₂O (3 × (5 × reaction volume)). The combined organic extracts were washed with brine (1 volume) and water (1 volume), then dried with MgSO₄ and concentrated in vacuo. If required, the alcohol product was purified by flash column chromatography.

11.2.2 APPLICATIONS OF YEAST GST-FUSION PROTEINS

Access to a large fraction of baker's yeast reductases in purified form allowed us to probe several important issues. For example, if a substrate is known to be reduced by whole yeast cells, the collection makes it possible to define which one(s) accept the substrate. In principle, this knowledge can be combined with amino acid sequence and protein structural information to understand better the interplay between protein structure and stereoselectivity, although this has been proven difficult in practice (*vide infra*). The collection of individual enzymes also opens the possibility of finding a biocatalyst with high stereoselectivity to meet the needs of a specific synthetic route. Since only a single reductase is used, products formed by competing yeast enzymes are eliminated, and this maximizes the chances of success. Moreover, keeping purified GST-fusion proteins on hand allows the screening tasks to be accomplished rapidly, of particular importance is the pharmaceutical industry, where time-to-market is a key driver in process development.

We have used a number of ketones to probe our collection of yeast GST-fusion proteins [18–21], and four representative examples are summarized below. Collectively, they illustrate the strengths and limitations of our current collection. In each case, it was possible to identify a yeast reductase that provided the desired stereoisomer; in three of the four cases, the enantiomer was also available by employing a different yeast enzyme. This is an important achievement, because difficulties in supplying both product enantiomers have traditionally been an Achilles heel of biocatalysis. Whereas, chemical catalysts can be reengineered to produce the enantiomeric product simply by inverting the stereochemistry of the chiral ligand field, this option is not practically available in biocatalysis. Instead, one must identify pairs of enantiocomplementary enzymes. Since the degree of enantiocomplementary will depend greatly on the substrate structure, it is necessary to screen the entire collection of enzymes for each new application, and a larger collection and simple assay methodologies maximize the chances of success.

11.2.2.1 Ethyl 4-Chloroacetoacetate

As noted above, the (*R*)-alcohol derived from ethyl 4-chloroacetoacetate is an important chiral building block in pharmaceutical and nutritional supplement synthesis. We screened 18 individual yeast reductases against this substrate, and the results are summarized in Figure 11.5. Five enzymes afforded only the (*S*)-alcohol within the limits of our detection, whereas nine yielded the homochiral (*R*)-alcohol. The remaining four enzymes displayed limited stereoselectivity.

Several interesting points emerged from these results. First, at least for our current collection of yeast reductases, **1b** is a “universal substrate” accepted by every enzyme. One would, therefore, expect that using this ketone to assess reductase activity in yeast protein fractions would reveal all 18 dehydrogenases in our collection. In fact, only four were found [9,10]. Presumably, the “missing” reductases are produced at low levels under the growth conditions used to produce yeast cells commercially. Using a genomic, rather than a bio-chemical, approach to identifying potentially useful enzymes side-steps problems of protein expression levels under physiological conditions.

The second general lesson from the studies of **1b** is that most yeast reductases, if they accept a given substrate, catalyze reactions with very high enantioselectivities. This reinforces the notion that the mixtures of products observed from reductions using whole yeast cells are primarily due to simultaneous participation by multiple reductases with opposing stereoselectivities. This demonstrates the very important practical benefits of examining individual reductases, rather than relying on whole organisms.

It is also noteworthy that we observed no simple correlation between stereoselectivity and amino acid sequence. For example, the high sequence similarity between the six yeast aldose

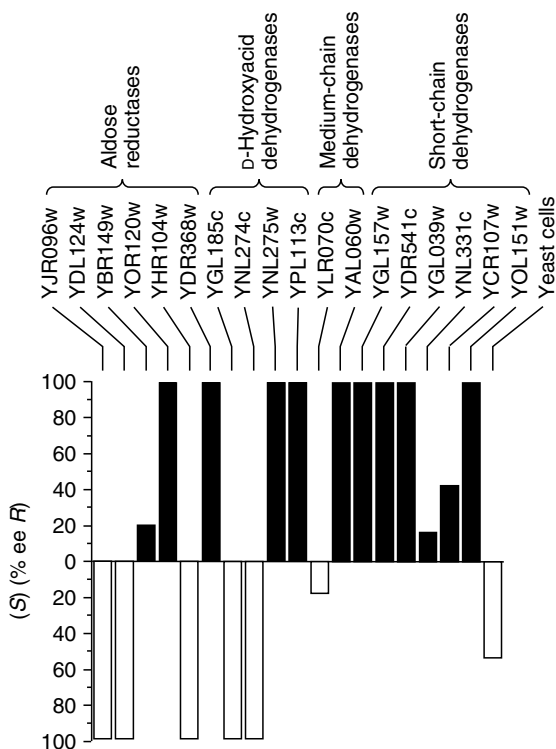


FIGURE 11.5 Reduction of ethyl 4-chloroacetoacetate by GST-fusion proteins. Systematic gene names are shown above, and enzymes are grouped by superfamily. White bars indicate reactions that proceed with predominant (*S*)-selectivity while those with predominant (*R*)-selectivity are represented by black bars.

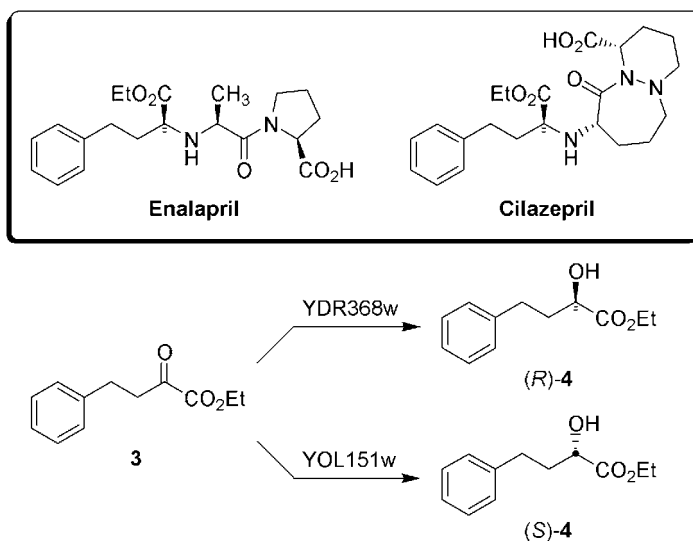
reductases likely indicates that all share the same overall structure. Despite this similarity, three yield only the (*S*)-alcohol and two produce the (*R*)-product. This underscores the difficulties in predicting the outcomes of enzymatic reactions involving unnatural substrates, and it is one of the major reasons that we favor simple, rapid screening approaches to determine these outcomes empirically. Our results also make it clear that when choosing whether or not to add a new protein to the dehydrogenase collection, high sequence similarity to existing members should not automatically disqualify a candidate.

11.2.2.2 Ethyl 4-Phenyl-2-Oxobutyrate

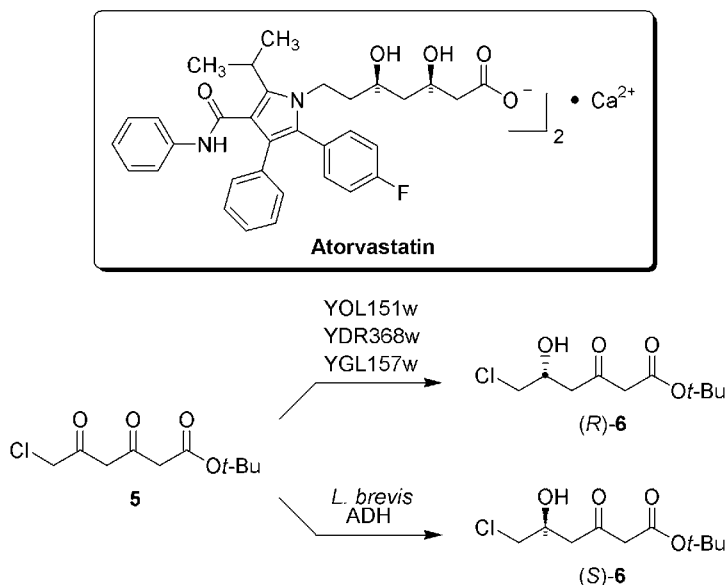
Alcohol (*R*)-4 is an important intermediate in commercial routes to a number of angiotensin converting enzyme (ACE) inhibitors such as Enalapril and Cilazapril, used clinically to regulate blood pressure (Scheme 11.2). A large number of synthetic approaches to (*R*)-4 have been published [18]. In an effort to find a direct route to (*R*)-4 from commercially available **3**, we screened our collection of isolated yeast dehydrogenases (as GST-fusion proteins) and discovered that the YDR368w enzyme afforded (*R*)-4 in 97% ee [18]. By contrast, the short-chain dehydrogenase encoded by the YOL151w yielded (*S*)-4 in 90% ee. While commercial interest in this enantiomer is much lower, the result further illustrates the stereochemical diversity of individual enzymes found in a single organism. These bioconversions were also successfully carried out with engineered *E. coli* strains overexpressing the yeast dehydrogenases.

11.2.2.3 Atorvastatin Building Block

The cholesterol-lowering drug, Atorvastatin, has been a top-selling pharmaceutical product over the past several years (Scheme 11.3). Not surprisingly, its synthesis in homochiral form has attracted a great deal of attention. The pyrrole and most of the flanking groups are achiral; however, the heptanoate side-chain contains two secondary hydroxyl groups whose stereochemistry must be controlled. This was the portion that attracted our interest. Our efforts were not aimed at developing a process to unseat the current manufacturing route, but instead to learn whether a chemoenzymatic approach could deliver all possible stereoisomer for this type of structure. Beyond its intrinsic academic appeal, synthesizing all potential



SCHEME 11.2



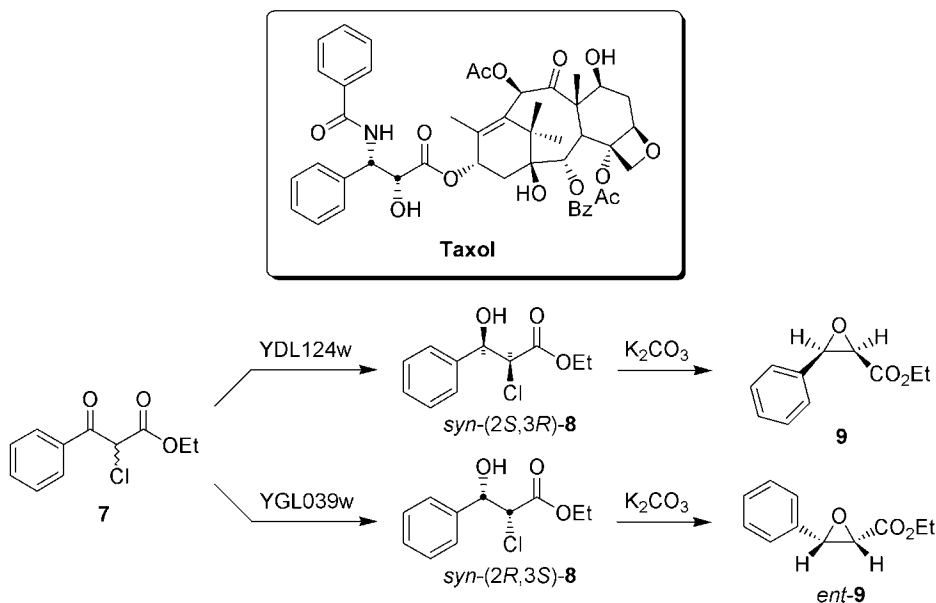
SCHEME 11.3

isomers of a target is required in early drug development and toxicology studies so that their biological activities can be profiled completely. In these applications, high volumetric productivity is not necessary; instead, high stereoselectivity and rapid enzyme discovery are keys to success.

We identified (*R*)- and (*S*)-**6** as our targets for producing all four stereoisomers of the key Atorvastatin building block since diastereoselective chemical transformations can use the configuration of the δ -hydroxyl to afford either the *syn*- or *anti*-diol (Scheme 11.3). The key is to introduce the first asymmetric center. Müller and coworkers identified an alcohol dehydrogenase from *Lactobacillus brevis* that enantio- and regioselectively reduced β,δ -diketo ester **5** to (*S*)-**6** [22,23]. Whole cells of baker's yeast were originally described as the best biocatalyst for producing (*R*)-**6**; however, the modest enantioselectivity observed (48% ee) prompted a search for better alternatives. In collaboration with the Müller group, we explored two complementary strategies. By systematically altering reaction conditions, it was possible to increase the enantiomeric purity of (*R*)-**6** to 94% ee when whole yeast cells were used as the biocatalyst [20]. Alternatively, screening individual yeast dehydrogenases as GST-fusion proteins revealed that three (encoded by the YOL151w, YDR368w and YGL157w genes) produced (*R*)-**6** in >96% ee. These reactions could also be carried out on gram scales with no erosion of stereoselectivity.

11.2.2.4 Taxol Side-Chain Precursor

Taxol has proven highly effective against certain ovarian and breast cancers (Scheme 11.4). The key step in the commercial route to this compound involves coupling an advanced precursor of the terpene core isolated from natural sources with the *N*-benzoyl-phenylisoserine side-chain prepared chemically [24]. Because of its small size, dense functionality and potential economic value, the Taxol side-chain has attracted a great deal of synthetic attention [25]. While few of these are likely to compete directly with the currently practiced commercial route, the molecule has provided an important test-bed for methods in asymmetric synthesis.



SCHEME 11.4

We envisioned a concise approach to the Taxol side-chain ethyl ester that would rely on an asymmetric reduction of α -chloro- β -keto ester **7** as the key step (Scheme 11.4) [26]. The highly acidic α -proton allows facile enol formation and reprotonation to occur, even at neutral pH values, so that **7** is racemic under the reduction conditions. This allows a dynamic kinetic resolution to be carried out, provided that the reduction methodology is both diastereo- and enantioselective. An early attempt to use whole yeast cells for stereoselective reduction of **7** yielded a disappointing mixture of diastereomers; we hoped that substituting individual yeast reductases might be more successful. Screening our collection of yeast GST-fusion proteins uncovered two with the desired properties. The dehydrogenase encoded by the YDL124w gene gave *syn*-(2*S*,3*R*)-**8** as the only observable product. This is the stereoisomer required to complete the synthesis of the natural enantiomer of the Taxol side-chain, and its absolute configuration was confirmed by conversion to the known *cis*-epoxide **9**. We also discovered that the protein product of the YGL039w gene afforded the enantiomeric *syn*-alcohol **8** as the major product (90% of the total). Unfortunately, it was not possible to separate the diastereomeric chlorohydrins from this reduction chromatographically; however, after cyclization to epoxide *ent*-**9**, the diastereomers could be separated cleanly. We have used both **9** and *ent*-**9** to produce the natural and unnatural enantiomers of the Taxol side-chain ethyl ester, respectively. This sequence illustrates how judicious choices of biocatalysts allow a common starting material to be converted into both target enantiomers without the need for resolution.

11.2.3 LARGE-SCALE REDUCTIONS

All of the efforts described above were focused on identifying yeast reductases with suitable substrate- and stereoselectivities toward specific ketones. Discovering the most appropriate enzyme(s), however, solves only half of the problem. Employing these conversions in synthetic routes demands that grams (or kilograms) of product can be prepared. One advantage of our strategy is that all reductases are produced by a common promoter in the same host cell. This simplifies process development.

Substituting whole *E. coli* cells that overexpress a yeast reductase for the corresponding purified GST-fusion protein eliminates the time- and resource-consuming protein purification steps. This in turn allows reductions to be carried out on larger (multiliter) scales. We originally used growing cultures of engineered *E. coli* cells to carry out reductions [27]. While these were successful, the volumetric productivities were relatively low since the cultures could be used for only a relatively short period of time. Early in the reactions, there were relatively few cells per unit volume, and this limited catalytic activity. As the cultures reached stationary phase, the bioconversions slowly ceased.

Based on the above criteria, we sought conditions that would allow a high concentration of engineered *E. coli* cells to be present during the reduction while not allowing the culture to enter stationary phase. This was accomplished by first growing the engineered *E. coli* cells under inducing conditions to load the cells with the reductase of interest. After they had been collected by centrifugation, the cells were resuspended in a minimal salts medium lacking a reduced nitrogen source. Solutions of glucose (to provide a source of NADPH by cellular metabolism) and the ketone substrate were added by pumps. This two-step approach was originally devised for an enzymatic Baeyer–Villiger oxidation [28], but it also proved very useful for NADPH-dependent ketone reductions [29].

We have used this technique for preparing the chlorohydrins (**8** and its enantiomer) required for our Taxol side-chain synthesis. As we have observed for many hydrophobic substrates, high concentrations of **7** were toxic and dramatically retarded *E. coli* cell growth. However, using fed-batch conditions, several grams of optically pure chlorohydrin could be obtained from a lab-scale 5 L fermenter [26].

11.3 SUMMARY AND FUTURE OUTLOOK

Our experience in cloning, expressing and screening a large fraction of the dehydrogenases from baker's yeast has yielded several useful lessons. First, it has demonstrated how the performance of yeast reductions can be increased dramatically by focusing on individual enzymes. In contrast to earlier strategies that attempted to minimize the catalytic activities of undesirable yeast enzymes by including inhibitors in reductions by whole yeast cells, or by substituting strains with single gene knockouts, employing pure yeast reductases very often yields stereochemically pure alcohols. In addition, we have shown that even a modest collection of approximately 20 reductases culled from a single genome is sufficiently large to address many synthetic needs. It was gratifying to discover that in most cases, enantio-complementary enzymes could be identified. Finally, our experience has shown that biocatalyst and discovery and process development can be accelerated significantly by screening cloned proteins in pure form. Even without the use of robotic assistance, screening reactions can be carried out rapidly and answers obtained in less than 48 h. Once the desired proteins have been identified, the corresponding genes are also immediately known, which facilitates further reaction engineering by altering overexpression levels, by moving the genes to more process-friendly hosts, etc.

While it might be supposed that proteins with high sequence similarities would have similar substrate- and stereoselectivities, our results argue that this is not necessarily the case. This is an important reason that we favor empirical screening to find useful enzymes for a given substrate, rather than trying to predict behavior on the basis of computer modeling. For nearly all of the enzymes investigated here, neither the physiological substrates nor the three-dimensional structures of the yeast reductases are known, so that little experimental data is available to guide computer modeling approaches. We are also reluctant to use amino acid similarity to existing library members as a basis for rejecting potential library additions since their properties may actually be quite novel.

11.3.1 JUDGING COMPLETENESS IN A COLLECTION

There are two ways to judge whether a given collection of enzymes is “complete.” On the one hand, if a library provides a solution to every synthetic problem investigated, it can be considered sufficiently large. Given the infinite number of potential substrates, this is obviously an impossible goal to achieve in practice. It might therefore be more reasonable to ask how many enzymes are required to fulfill a significant fraction, for example 80%, of the synthetic demands. This is a key question when deciding an adequate level of resources devoted to discovering, cloning and isolating the fusion proteins that make up a library. In the case of yeast dehydrogenases, even our modest collection of approximately 20 members has generally been up to the task. There are exceptions, however, and we are currently addressing these needs by adding additional proteins (*vide infra*).

The second way to judge “completeness” in an enzyme library is to ask whether they collectively yield all of the products observed when a substrate is tested against whole cells of the organism from which the genes were cloned. In our example of baker’s yeast dehydrogenases, it is clear that we have not yet met this criterion for completeness. For example, none of the yeast enzymes in our current collection produced (S)-**6**, even though this was a major product from reactions involving whole yeast cells. Reductions of α -chloro- β -keto ester **7** provide another example, where whole yeast cells produce an alcohol diastereomer that is not observed when **7** is screened against our 20 library members [30]. This problem also highlights an important challenge in using a bioinformatics approach to identifying all of the relevant enzymes in a given organism. While it is probable that (S)-**6** originates from one or more of the enzymes listed in Table 11.1 that are not yet members of our yeast dehydrogenase library, we cannot discount the possibility that an enzyme with little or no sequence similarity to well-characterized dehydrogenases and carbonyl reductases is responsible. Such an enzyme would not appear in Table 11.1. The only recourse would be to examine every ORF in the yeast genome until every protein involved has been identified, the original purpose for which the library of yeast GST-fusion proteins was developed [17].

11.3.2 EXPANDING THE DEHYDROGENASE COLLECTION

In practice, the most common problem with the existing GST-fusion protein library was that not every alcohol stereoisomer could be prepared, particularly in the case of α -substituted β -keto esters. How should the collection be expanded to solve these problems? Given limited resources, the key is to focus the search for new enzymes on the subset most likely to broaden the range of available products. One possibility is to include the additional *S. cerevisiae* reductase candidates listed in Table 11.1. When “missing” products are known to be produced by reductions with whole yeast cells, this strategy has a good chance of succeeding. Because only a subset of genes are expressed in commercial yeast cells, this approach is also likely to provide additional products beyond those anticipated from whole-cell results. It is also possible to expand our current dehydrogenase collection by incorporating genes from other organisms. In cases, where gene sequences are known (either by cloning individual proteins or by sequencing whole genomes), these can be expressed as GST-fusion proteins in the same manner as used for the existing collection. On the other hand, when whole cells of an unsequenced organism are known to carry out the reaction of interest, more laborious approaches (protein isolation, amino acid microsequencing, etc.) are required. We favor this strategy only for the most difficult cases. Our first choice would be to examine already-known genes. Given the diversity in substrate- and stereoselectivities exhibited by proteins with highly similar sequences, we believe that this will provide synthetic solutions in most cases.

11.3.3 WIDER APPLICABILITY AND LIMITATIONS

We have used the *S. cerevisiae* genome and the asymmetric ketone reductions to provide the proof-of-principle for our approach. We deliberately designed the strategy so that it could be applied to any genome and reaction of interest. All that is required is to have sequences from one or more proteins known to catalyze a particular reaction, which can be used to probe sequence databases and provide library candidates. Creating expression clones for GST-fusion proteins is the most labor-intensive phase of the project. While our original library of *E. coli* overexpression plasmids was constructed by stepwise “cut-and-paste” molecular biology techniques, we have more recently developed recombination-based cloning methods that allow direct incorporation of PCR products into the fusion protein expression vector [31]. This has dramatically shortened the time required to isolate purified proteins and expedites screening. It should be noted that a variety of other expression systems and purification “handles” are available; using *E. coli* and fusion with GST was an expedient choice based on the methods used to construct the original genome-wide library from *S. cerevisiae*.

While we believe that our approach has a number of advantages, it is also important to keep several limitations in mind. First, success in identifying candidate proteins by genome mining depends on access to sequences of proteins known to carry out the desired chemical conversion (“bait” sequences). Including members of multiple sequence families as sequence probes increases both the number and the variety of candidates that may exhibit the desired catalyst characteristics. In some cases, however, no suitable “bait” sequences exist, either because no enzyme catalyst for the reaction of interest has been identified or because the proteins have not yet been sequenced. This precludes genome mining. The solution will be to screen whole cells for the reaction of interest, then to identify the enzyme(s) and the gene(s) involved. Given the very large number of whole genomes sequences available, having even one “bait” sequence from such an approach will likely result in a number of library candidates.

A second limitation on phenotypic screening of proteins is that they must be catalytically active. Problems with low expression, including body formation and misfolding can be minimized by expressing library proteins in suitable hosts. To date, we have relied on *E. coli* because of its rapid growth in inexpensive media coupled with efficient overexpression systems. Other hosts might be more appropriate for different classes of enzymes, however, particularly those requiring specific posttranslational modifications or cofactors that are not available in the *E. coli* cytoplasm.

Finally, protein structure may also limit the breadth of enzymes amenable to our library approach; membrane-bound enzymes must be purified and screened in the presence of detergents and those that are only active as part of multienzyme complexes are handled in these forms. To date, we have not made significant efforts to include these types of “difficult” proteins in our library. We always observe several enzyme “hits” for a given substrate, inactive or poorly expressed proteins are simply dropped from further consideration. In our view, enlarging the library by adding well-expressed and stable proteins is more resource-efficient than spending inordinate efforts to make even poorly behaved proteins available. Hampered by these problems, such enzymes are unlikely to be used as the basis for a scalable bioprocess.

ACKNOWLEDGMENTS

The author’s research in this area has been generously supported by the National Science Foundation (CHE-0130315) and Great Lakes Fine Chemicals. We are also grateful to Mark Martzen for sharing the original yeast GST-fusion protein clones. The real credit for the work belongs to the students and coworkers whose hard work has reduced the ideas to practice.

REFERENCES

1. Patel, R.N., Enzymatic synthesis of chiral intermediates for drug development, *Adv. Synth. Catal.*, 343, 527–546, 2001.
2. Breuer, M., Ditrach, K., Habicher, T., et al., Industrial methods for the production of optically active intermediates, *Angew. Chem. Int. Ed. Engl.*, 43, 788–824, 2004.
3. Grasa, G.A., Singh, R., and Nolan, S.P., Transesterification/acylation reactions catalyzed by molecular catalysts, *Synthesis*, 971–985, 2004.
4. Nakamura, K., Yamanaka, R., Matsuda, T., et al., Recent developments in asymmetric reduction of ketones with biocatalysts, *Tetrahedron Asymmetry*, 14, 2659–2681, 2003.
5. Santaniello, E., Ferraboschi, P., Grisenti, P., et al., The biocatalytic approach to the preparation of enantiomerically pure chiral building blocks, *Chem. Rev.*, 92, 1071–1140, 1992.
6. Santaniello, E., Ferraboschi, P., and Manzocchi, A., Recent advances on bioreductions mediated by baker's yeast and other microorganisms, in *Enzymes in Action. Green Solutions for Chemical Problems*, Zwanenburg, B., Mikolajczyk, M., Kielbasinski, P., Eds., Kluwer Academic, Dordrecht, The Netherlands, 2000, pp. 95–115.
7. Seebach, D., Sutter, M.A., Weber, R.H., et al., Yeast reduction of ethyl acetoacetate: (S)-(+)-3-hydroxybutanoate, *Org. Synth.*, 63, 1–9, 1985.
8. Zhou, B.-N., Gopalin, A.S., VanMiddlesworth, F., et al., Stereochemical control of yeast reductions. 1. Asymmetric synthesis of L-carnitine, *J. Am. Chem. Soc.*, 105, 5925–5926, 1983.
9. Shieh, W.-R., Gopalin, A.S., and Sih, C.J., Stereochemical control of yeast reductions. 5. Characterization of the oxidoreductases involved in the reduction of β -keto esters, *J. Am. Chem. Soc.*, 107, 2993–2994, 1985.
10. Nakamura, K., Kawai, Y., Nakajima, N., et al., Stereocontrol of microbial reduction. 17. A method for controlling the enantioselectivity of reductions with bakers' yeast, *J. Org. Chem.*, 56, 4778–4783, 1991.
11. Baraldi, P.T., Zarbin, P.H.G., Vieira, P.C., et al., Enantioselective synthesis of (R)- and (S)-2-methyl-4-octanol, the male-produced aggregation pheromone of *Curculionidae* species, *Tetrahedron Asymmetry*, 13, 621–624, 2002.
12. Schweizer, E. and Bolling, H., A *Saccharomyces cerevisiae* mutant defective in saturated fatty acid biosynthesis, *Proc. Natl. Acad. Sci. USA*, 67, 660–666, 1970.
13. Kühn, L., Castorph, H., and Schweizer, E., Gene linkage and gene-enzyme relations in the fatty-acid-synthetase system of *Saccharomyces cerevisiae*, *Eur. J. Biochem.*, 24, 492–497, 1972.
14. Rodriguez, S., Kayser, M.M., and Stewart, J. D., Improving the stereoselectivity of bakers' yeast reductions by genetic engineering, *Org. Lett.*, 1, 1153–1155, 1999.
15. Rodriguez, S., Kayser, M.M., and Stewart, J.D., Highly stereoselective reagents for β -keto ester reductions by genetic engineering of bakers' yeast, *J. Am. Chem. Soc.*, 123, 1547–1555, 2001.
16. Stewart, J.D., Rodriguez, S., and Kayser, M.M., Cloning, structure and activity of ketone reductases from bakers' yeast, in *Enzyme Technology for Pharmaceutical and Biotechnological Applications*, Kirst, H.A., Yeh, W.-K., and Zmijewski, M.J., Eds., Marcel Dekker, New York, 2001, pp. 1–208.
17. Martzen, M.R., McCraith, S.M., Spinelli, S.L., et al., A biochemical genomics approach for identifying genes by the activity of their products, *Science*, 286, 1153–1155, 1999.
18. Kaluzna, I., Andrew, A.A., Bonilla, M., et al., Enantioselective reductions of ethyl 2-oxo-4-phenylbutyrate by *Saccharomyces cerevisiae* dehydrogenases, *J. Mol. Catal., B Enzym.*, 17, 101–105, 2002.
19. Kaluzna, I.A., Matsuda, T., Sewell, A.K., et al., A systematic investigation of *Saccharomyces cerevisiae* enzymes catalyzing carbonyl reductions, *J. Am. Chem. Soc.* 126, 12827–12832, 2004.
20. Wolberg, M., Kaluzna, I.A., Müller, M., et al., Regio and enantioselective reduction of *t*-butyl 6-chloro-3,5-dioxohexanoate with baker's yeast, *Tetrahedron Asymmetry*, 15, 2825–2828, 2004.
21. Kaluzna, I.A., Feske, B.D., Wittayanan, W., et al., Stereoselective, biocatalytic reductions of α -chloro- β -keto esters, *J. Org. Chem.*, 70, 342–345, 2005.
22. Wolberg, M., Hummel, W., and Müller, M., Biocatalytic reduction of β,δ -diketo esters: a highly stereoselective approach to all four stereoisomers of a chlorinated β,δ -dihydroxy hexanoate, *Chem. Eur. J.*, 7, 4562–4571, 2001.

23. Wolberg, M., Ji, A., Hummel, W., and Müller, M., Enzymatic reduction of hydrophobic β,δ -diketo esters, *Synthesis*, 937–942, 2001.
24. Wall, M.E. and Wani, M.C., Paclitaxel—from discovery to clinic, *ACS Symp. Ser.*, 583, 18–30, 1995.
25. Borah, J.C., Gogoi, S., Boruwa, J., et al., A highly efficient synthesis of the C-13 side-chain of taxol using Shibasaki's asymmetric Henry reaction, *Tetrahedron Lett.*, 45, 3689–3691, 2004.
26. Feske, B.D., Kaluzna, I.A., and Stewart, J.D., Enantiodivergent, biocatalytic routes to both taxol C-13 side-chain enantiomers, *J. Org. Chem.*, 70, 9654–9657, 2005.
27. Rodriguez, S., Schroeder, K.T., Kayser, M.M., et al., Asymmetric synthesis of β -hydroxy esters and α -alkyl- β -hydroxy esters by recombinant *Escherichia coli* expressing enzymes from bakers' yeast, *J. Org. Chem.*, 65, 2586–2587, 2000.
28. Walton, A.Z. and Stewart, J.D., An efficient enzymatic Baeyer–Villiger oxidation by engineered *Escherichia coli* cells under non-growing conditions, *Biotechnol. Prog.*, 18, 262–268, 2002.
29. Walton, A.Z. and Stewart, J.D., Understanding and improving NADPH-dependent reactions by non-growing *Escherichia coli* cells, *Biotechnol. Prog.*, 20, 403–411, 2004.
30. Cabon, O., Buisson, D., Larcheveque, M., et al., The microbial reduction of 2-chloro-3-oxoesters, *Tetrahedron Asymmetry*, 6, 2199–2210, 1995.
31. Bougioukou, D. and Stewart, J.D., unpublished results, 2005.

12 Immobilization of Enzymes as Cross-Linked Enzyme Aggregates: A Simple Method for Improving Performance

Roger A. Sheldon

CONTENTS

12.1	Introduction	351
12.2	Immobilization by Cross-Linking	353
12.3	Cross-Linked Enzyme Aggregates.....	353
12.4	Lipase Cleas: Hyperactivation and the Effect of Additives.....	354
12.5	Clea Activities in “Anhydrous” Organic Media	355
12.6	Particle Size and Mass Transfer Effects	356
12.7	Cross-Linking Agents.....	357
12.8	Scope of the Methodology	358
12.9	Combi Cleas and Catalytic Cascade Processes.....	359
12.10	Conclusions and Prospects.....	360
	References	360

12.1 INTRODUCTION

Currently much attention is focused on the application of atom efficient catalytic methodologies—heterogeneous, homogeneous, and enzymatic—in organic synthesis, both in industry and in academe [1]. The ultimate goal is the development of green, sustainable technologies for the manufacture of (fine) chemicals. In this context, biocatalytic methodologies have many potential benefits compared with traditional organic syntheses. They generally employ mild reaction conditions (ambient temperature and pressure at physiological pH) and afford high chemo-, regio-, and stereoselectivities. Furthermore, enzymatic syntheses generally involve few steps by obviating the need for protection and deprotection steps, and by avoiding the use of environmentally unattractive organic solvents. This affords syntheses that are short, less energy intensive, and generates less waste; hence are both environmentally and economically more attractive.

The time is ripe for the widespread application of biocatalysis, using whole cells or isolated enzymes in industrial organic synthesis [2–16]. According to a 2002 review [2], there are more than 130 biotransformations currently performed in an industrial scale, mostly in the manufacture of pharmaceutical intermediates and other fine chemicals. Advances in recombinant

DNA techniques [17] have made it, in principle, possible to produce any enzyme for a commercially acceptable price and widespread sourcing of enzymes from extreme environments and genomic DNA data has significantly expanded the pool of available enzymes [18]. Furthermore, advances in protein engineering have made it possible, using techniques such as site-directed mutagenesis and *in vitro* evolution by means of DNA shuffling [19–22], to manipulate enzymes such that they exhibit the desired properties: substrate specificity, activity, selectivity, productivity, stability, pH profile, and so forth. Nonetheless, the commercialization of many enzymatic processes is often hampered by the lack of operational stability of many enzymes, coupled with their relatively high price. This impediment can generally be overcome if one can find an effective method for their immobilization [7,23–25]. If successful, this not only results in improved operational stability but also allows for facile separation and reuse of the enzyme, and simplifies downstream processing.

Conceptually, immobilization methods can be conveniently divided into three types: (a) binding to a support (carrier), (b) encapsulating in an inorganic or organic polymeric gel, (c) cross-linking of the protein molecules [23]. These three types can be further subdivided on the basis of the technique used for binding, encapsulating, or cross-linking as shown in Figure 12.1.

A distinct disadvantage of carrier-bound enzymes, whether they involve binding to, or encapsulation in, a carrier, is the dilution of catalytic activity resulting from the introduction of a large proportion of noncatalytic mass, generally ranging from 90 to >99% of the total mass [23]. This inevitably leads to lower volumetric and space-time yields, and lower catalyst productivities. Attempts to achieve high enzyme loadings usually lead to loss of activity due to leaching. The third type of immobilization, cross-linking of enzyme molecules with a bifunctional cross-linking agent, most commonly glutaraldehyde, does not suffer from this disadvantage. The molecular weight of the cross-linking agent is negligible compared with that of the enzyme and the resulting biocatalyst essentially comprises 100% active enzyme.

Whichever method is used, an ideal industrial immobilized enzyme has to meet several criteria, such as being recyclable, broadly applicable, cost-effective, and safe for use. Furthermore, deactivation is not always avoidable. Consequently, the disposal of deactivated immobilized enzymes also has to be taken into account, especially for a large-scale industrial production that might require multihundred tons of a carrier-bound immobilized enzyme on an annual basis, for example, the immobilized penicillin G acylase needed for the production of semisynthetic β -lactam antibiotics.

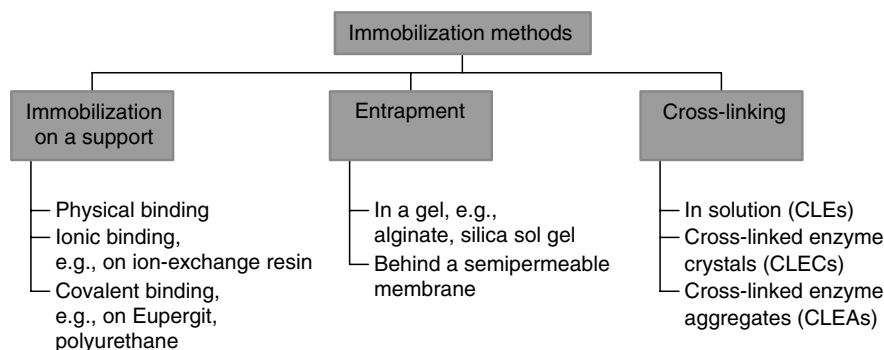


FIGURE 12.1 Methods for immobilization of enzymes.

12.2 IMMOBILIZATION BY CROSS-LINKING

The technique of protein cross-linking by the reaction of glutaraldehyde with reactive NH_2 groups on the protein surface was initially developed in the 1960s [24]. However, this method of producing cross-linked enzymes (CLEs) had several drawbacks, such as low activity retention, poor reproducibility, low mechanical stability, and difficulties in handling the gelatinous CLEs. Mechanical stability and ease of handling could be improved by cross-linking the enzyme in a gel matrix or a carrier, but this led to the disadvantageous dilution of catalytic activity. Consequently, in the late 1960s, emphasis switched to carrier-bound enzymes, which became the most widely used industrial methodology for enzyme immobilization for the next three decades.

The cross-linking of a crystalline enzyme by glutaraldehyde was first described by Quijcho and Richards in 1964 [25]. Their main objective was to stabilize enzyme crystals for x-ray diffraction studies, but they also showed that catalytic activity was retained. The use of cross-linked enzyme crystals (CLECs) as industrial biocatalysts was pioneered by scientists at Vertex Pharmaceuticals in the early 1990s [26], and subsequently commercialized by Altus Biologics [27–30]. The initial studies were performed with CLECs of thermolysin, of interest in the manufacture of aspartame, but the method was subsequently shown to be applicable to a broad range of enzymes. CLECs proved significantly more stable to denaturation by heat, organic solvents, and proteolysis than the corresponding soluble enzyme or lyophilized (freeze-dried) powder. CLECs are robust, highly active immobilized enzymes of controllable particle size, varying from 1 to 100 μm . Their operational stability and ease of recycling, coupled with their high catalyst and volumetric productivities, render them ideally suited for industrial biotransformations.

12.3 CROSS-LINKED ENZYME AGGREGATES

An inherent disadvantage of CLECs is the need to crystallize the enzyme, which is often a laborious procedure requiring enzymes of high purity. Consequently, we reasoned that comparable results could possibly be achieved by simply precipitating the enzyme from aqueous solution, using standard techniques, and cross-linking the resulting physical aggregates of enzyme molecules (Figure 12.2). This indeed proved to be the case and led to the development of a new family of cross-linked enzymes, which we have called cross-linked enzyme aggregates (CLEA). Protein purification by precipitation from aqueous solution with, e.g., ammonium sulfate or polyethylene glycol, is the most frequently used primary method of protein purification, and the preparation of CLEAs can be easily integrated into a purification protocol as it does not require a highly purified enzyme.

It is well known [31] that the addition of salts, organic solvents, or nonionic polymers to aqueous solutions of proteins leads to their precipitation as physical aggregates of protein molecules without perturbation of their tertiary structure, that is without denaturation. These

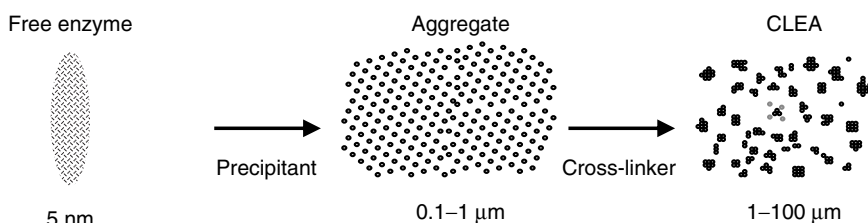
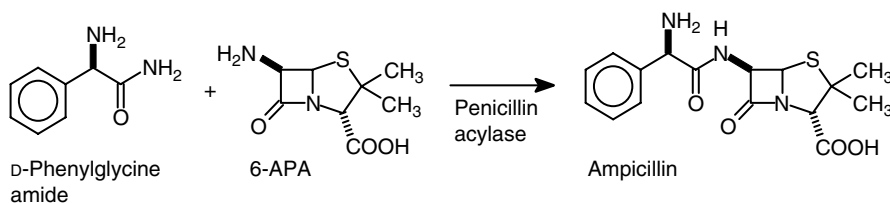


FIGURE 12.2 Preparation of a CLEA.

solid aggregates are held together by noncovalent bonding, and readily collapse and redissolve when dispersed in water. Cross-linking of these physical aggregates produces CLEAs that are rendered permanently insoluble while maintaining the preorganized superstructure of the aggregates, and, hence their catalytic activity. Initial studies [32,33] focused on the synthesis of CLEAs from penicillin G acylase (penicillin amidohydrolase, E.C. 3.5.1.11), an industrially important enzyme used in the synthesis of semisynthetic penicillin and cephalosporin antibiotics [34]. The free enzyme has limited thermal stability and low tolerance to organic solvents, making it an ideal candidate for stabilization as a CLEA. Indeed, penicillin G acylase CLEAs, prepared by precipitation with, for example, ammonium sulfate or *tert*-butanol, proved to be effective catalysts for the synthesis of ampicillin according to Reaction 12.1 [35].



Reaction 12.1

The CLEA exhibited a synthesis/hydrolysis ratio (S/H) comparable with that of the commercial catalyst, PGA-450 (penicillin G acylase immobilized on poly-acrylamide), and substantially higher than that of the penicillin G acylase CLEC (Table 12.1). This suggests that diffusional limitations are more severe in the CLEC than in the CLEA or the PGA-450. Remarkably, the productivity of the CLEA was even higher than that of the free enzyme that it was made from and substantially higher than that of the CLEC. Not surprisingly, the productivity of the commercial catalyst was very low, a reflection of the fact that it mainly consists of noncatalytic ballast in the form of polyacrylamide carrier. Analogous to the corresponding CLECs, the penicillin G acylase CLEAs also maintained their high activity in organic solvents [35–37].

12.4 LIPASE CLEAS: HYPERACTIVATION AND THE EFFECT OF ADDITIVES

We next turned our attention to an investigation of the effect of various parameters, such as the precipitant and the addition of additives, and surfactants and crown ethers, on the

TABLE 12.1
Ampicillin Synthesis Catalyzed by Different Penicillin G Acylase Preparations

Biocatalyst	Conversion (%)	S/H ^a	V _{syn} (μmol/U/h) ^b	Relative Productivity ^c
Free enzyme	88	2.0	25.5	100
CLEC	72	0.71	39.6	39
T-CLEA ^d	85	1.58	38.2	151
PGA-450	86	1.56	15.6	0.8

^aSynthesis and hydrolysis molar ratio at the conversion listed.

^bInitial reaction rate.

^cRelative productivity at maximum conversion, free enzyme set at 100.

^dCLEA produced using *tert*-butanol as precipitant.

TABLE 12.2
Hyperactive Lipase CLEAs in Organic Medium

Lipase	Precipitant/Additive	Relative Reactivity (%)
<i>C. antarctica A</i>	(NH ₄) ₂ SO ₄ /SDS	900
<i>C. antarctica B</i>	DME	75
<i>P. alcaligenes</i>	(NH ₄) ₂ SO ₄	1200
<i>T. lanuginosus</i>	DME	82
<i>Rh. miehei</i>	(NH ₄) ₂ SO ₄ /SDS	250
<i>A. niger</i>	(NH ₄) ₂ SO ₄ /SDS	200
<i>C. rugosa</i>	(NH ₄) ₂ SO ₄ /TR	420

activities of CLEAs prepared from seven commercially available lipases [38]. The activation of lipases by additives, such as surfactants, crown ethers, and amines, is well documented and is generally attributed to the lipase induced to adopt a more active conformation [39]. We, therefore, reasoned that cross-linking of enzyme aggregates, resulting from precipitation in the presence of such an additive, would “lock” the enzyme in this more favorable conformation. Moreover, since the additive is not covalently bonded to the enzyme, the additive can subsequently be washed from the CLEA using, for example, an appropriate organic solvent.

Using this procedure, we succeeded in preparing a variety of hyperactive lipase CLEAs exhibiting activities even higher than the corresponding free enzyme, that is up to 12 times the activity of the free enzyme in the hydrolysis of ethyl octanoate in a 1,2-dimethoxyethane/water (95/5: v/v) mixture (Table 12.2) [39]. We also demonstrated that the experimental procedure for CLEA preparation could be further simplified by combining precipitation, either in the presence of additives or in the absence of additives, with cross-linking in a single operation [39].

Hence, the potential of the CLEA technology for preparing immobilized enzymes with high catalyst and volumetric productivities, in some cases exceeding those of the native enzymes that they were derived from, was firmly established. The method is exquisitely simple and can be performed with relatively impure enzyme preparations. Subsequent studies have been aimed at optimizing protocols with regard to parameters, such as temperature, pH, concentration, stirring rate, precipitant, additives, and cross-linking agent and exploring the scope of the technology (see later). The relative simplicity of the operation ideally lends itself to automation, e.g., using 96-well plates.

12.5 CLEA ACTIVITIES IN “ANHYDROUS” ORGANIC MEDIA

It is noteworthy that the CLEA derived from the most popular lipase, *Candida antarctica* lipase B (CaLB), showed only very moderate hyperactivation. Subsequent studies of CLEA preparations revealed that the optimum performance of lipase CLEAs observed in aqueous media could not be directly translated to organic media. Hence, the preparation of the CLEA was modified to allow for a better diffusion of substrates into the particles and to decrease the repulsion of organic solvents. Application of this modified “organic media technology” for

TABLE 12.3**Activities of Various Lipase B Formulations in Water and Organic Media**

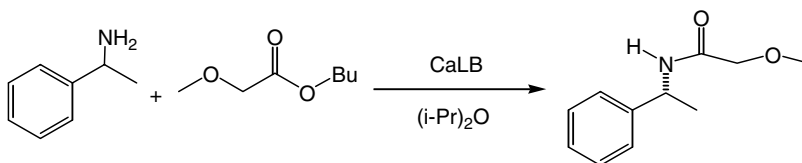
CaLB Preparation	Aqueous Activity ^a	Organic Activity ^b	Ratio Activity ^b
Native (lyophilized NOVO preparation)	22,000	—	—
Novozym 435	7,300	250	29
CLEA-AM ^c	38,000	50	760
CLEA-OM ^c	31,000	1,500	21

^aTributyrin: 5 vol% in 40 mM Tris buffer, pH 7.5, 40°C.

^bPhenylethylamine 41 mM, *n*-butyl methoxyacetate 34 mM, 12 mg/ml, molecular sieve 4A, 40°C.

^cAM = Developed for aqueous media, OM for organic media.

CLEA preparation dramatically improved the activity of CaLB CLEA in the enantioselective acylation of 1-phenethylamine in diisopropyl ether as solvent (Reaction 12.2). Table 12.3 shows a comparison of the results obtained with Novozym 435, the immobilized form of CaLB available from Novozymes and with CaLB CLEAs optimized for operation in water or organic solvents. Clearly, the optimized CaLB CLEAs have activities surpassing those of Novozym 435 in both aqueous and organic media. It is also worth noting that Novozym 435 cannot be recycled in water as the enzyme is leached from the surface (immobilization does not involve covalent bonding to the support).

**Reaction 12.2**

We have also shown that CaLB CLEAs exhibit superior activities, compared with standard organic solvents, in ionic liquids and supercritical carbon dioxide (unpublished results).

12.6 PARTICLE SIZE AND MASS TRANSFER EFFECTS

One major property of CLEAs, that was up to now relatively unexplored, is their particle shape and size, which obviously can have a direct effect on mass transfer limitations under operational conditions. The number of enzyme molecules and the way they are packed together in an aggregate can be expected to have a crucial influence on the activity of the aggregate as a whole. Hence, an understanding of the parameters which influence particle size and how to control them will pave the way for changing the CLEA from an interesting phenomenon into a mature, well-defined catalytic particle. Scanning electron microscopy showed a very uniform structure of the aggregates (see Figure 12.3 for *C. antarctica* lipase B). The diameter of the CLEA is about 1 μm with a small deviation. Taking an enzyme size for CaLB of $5 \times 5 \times 5 \text{ nm}$, a single CLEA particle contains a maximum 8×10^6 enzyme molecules. CLEAs can form larger clusters that do have mass-transport limitations, especially in fast UV-based assays. The size of these clusters can be up to 100 μm (Figure 12.3), making them visible to the naked eye. The number of CLEAs in a cluster is less uniform than the enzymes in an aggregate. It can vary from a few to a hundred thousand. Our previous findings with

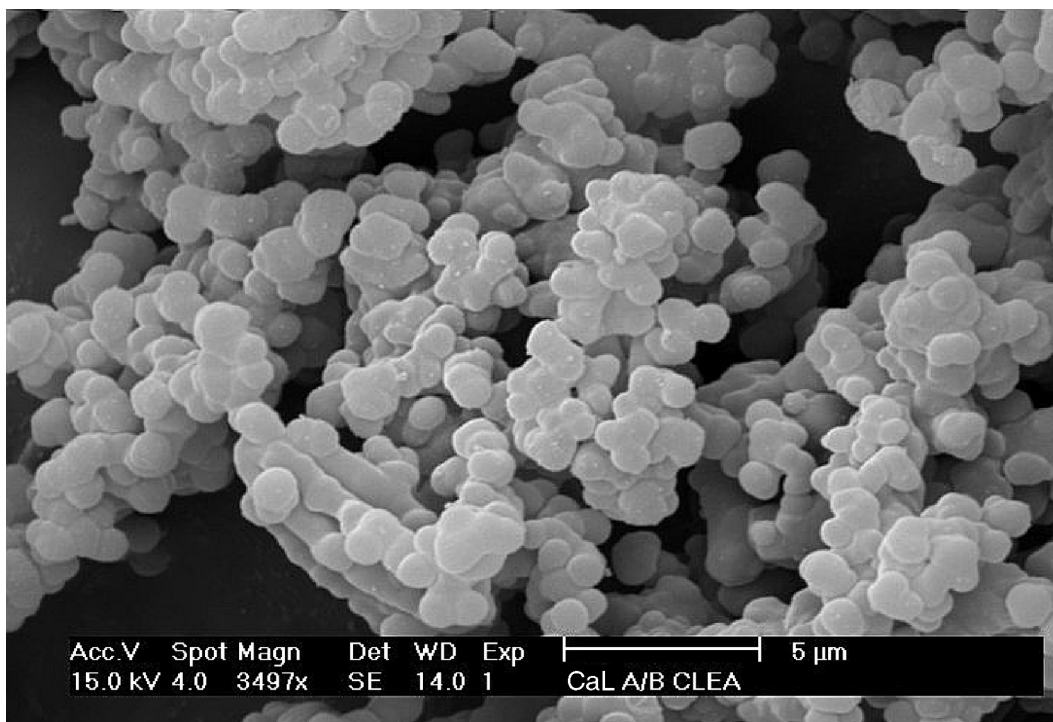


FIGURE 12.3 *Candida antarctica* lipase A/B CLEA, 1 CLEA particle can contain up to 8 million enzyme molecules (magnification 3500 \times).

laser scattering (data not shown) suggested that a variable number of enzyme molecules per aggregate are now rationalized as variable number of aggregates per cluster.

When a dispersed CLEA is assayed for activity, directly after dilution of the cross-link medium, a higher activity is observed than when the sample is centrifuged and redispersed. This treatment presumably does not disturb the individual CLEA and thereby the enzyme structure, but it does squeeze the CLEA particles close together, elevating mass-transport limitations to a noticeable level. Some differences between enzymes were observed: with CaLB very large and hydrophobic clusters were obtained, whereas with β -galactosidase well-dispersible suspensions were common. The most noticeable structural difference between these two enzymes is that β -galactosidase is extensively glycosylated while CaLB is not. Comparing activities of CaLB CLEA, found in the relatively fast hydrolysis of *p*-nitrophenyl propionate measured by UV/Vis absorption, with slower hydrolysis of triacetin (monitored by titration) the mass-transport limitation was obvious. Compared with free enzyme, the first showed 35% activity recovery and the latter 177%. For β -galactosidase, however, activity recovery found in the hydrolysis of *p*-nitrophenyl- β -D-galactopyranoside and in lactose hydrolysis was the same. These two CLEAs emphasize the important effect of cluster formation on the apparent activity.

12.7 CROSS-LINKING AGENTS

Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities. However, we found that good results were not always obtained when using glutaraldehyde as the cross-linker. With some enzymes, e.g., nitrilases,

we observed low or no retention of activity. We were surmized that this total loss of activity might be caused by the reaction of cross-linker with amino acid residues, which are crucial for the activity of the enzyme. If this was so, then, we expected that inactivation would be particularly severe with glutaraldehyde owing to its high reactivity and small size, which allows it to penetrate the internal structure of the protein where it can react with amino acids that are essential for activity. If this was the problem then the solution was eminently simple: use a bulky cross-linking agent, which cannot access the internal surface of the protein. To this end, we employed bulky polyaldehydes obtained by periodate oxidation of dextrans [40], followed by reduction of the Schiff's base moieties with sodium borohydride, to form irreversible linkages. The activity retention of these CLEAs was generally much higher than that observed with CLEAs prepared using glutaraldehyde. Dramatic results were obtained with two nitrilases, one from *Pseudomonas fluorescens* and the other from the company, Biocatalytics (Biocatalytics 1004): it was observed that cross-linking with glutaraldehyde produced a completely inactive CLEA, while cross-linking with dextran polyaldehyde produced 50 to 60% activity retention (not optimized).

12.8 SCOPE OF THE METHODOLOGY

Having established the potential of the CLEA technology for preparing immobilized enzymes with high catalyst and volumetric activities, in some cases with activities significantly exceeding those of the native enzymes they were derived from, we set about investigating its scope. As noted earlier, the methodology essentially combines enzyme preparation (by precipitation) and immobilization into one step. We note, however, that if an impure sample containing a mixture of enzymes is used, this can lead to a CLEA containing more than one enzyme. We have used this to our advantage in the deliberate preparation of combi CLEAs, containing two or more enzymes, for use in multistep, biocatalytic cascade processes (see later).

We have shown that, by a suitable optimization of the procedure, which may differ from one enzyme to another, the CLEA methodology is applicable to essentially any enzyme, including cofactor dependent oxidoreductases and lyases in addition to a wide variety of hydrolases (Table 12.4).

For example, we have shown that a CLEA of β -galactosidase, which catalyzes the hydrolysis of lactose and is administered as "tolerase" to people suffering from lactose intolerance, was recycled with no loss of activity [41]. We have successfully prepared recyclable CLEAs from glucose oxidase and galactose oxidase (Schoevaart, in press). Guisan and

TABLE 12.4
Examples of Enzymes That Have Been Successfully Cleated

Hydrolases	Oxidoreductases	Lyases
Penicillin acylases (2)	Glucose oxidase	<i>R</i> - and <i>S</i> -Oxynitrilase
Aminoacylase	Galactose oxidase	Pyruvate decarboxylase
Pig liver esterase	Laccase	<i>R</i> -Deoxyribose aldolase (DERA)
Lipases (7)	Catalase	
Nitrilases (2)	Alcohol dehydrogenase	
Galactosidase	Formate dehydrogenase	
Proteases (2)		
Phytase		

coworkers [42] have recently pointed out that the CLEA technology has an additional benefit: it can stabilize the quaternary structures of multimeric enzymes. To demonstrate this, they prepared CLEAs from two tetrameric catalases [42]. The enzyme stability, which for the soluble enzyme is dependent on concentration, became independent of this parameter in the CLEA, which allowed for the use of low “concentrations” of catalase.

Another development is the inclusion of CLEAs in nonprotein material. Since CLEAs are an average factor 1000 bigger than free enzyme, enclosure into materials that would retain none or only low amounts of enzyme is now feasible [37,43].

12.9 COMBI CLEAS AND CATALYTIC CASCADE PROCESSES

Fine chemical syntheses generally involve multistep syntheses and the ultimate inefficiency is to combine these, preferably catalytic, steps into a one-pot multistep catalytic cascade process [44]. Indeed, this is truly emulating the metabolic pathways conducted in living cells by an elegant orchestration of a series of biocatalytic steps into an exquisite multicatalyst cascade, without the need for separation of intermediates. Catalytic cascade processes have numerous potential benefits: fewer unit operations, less reactor volume, and higher volumetric and space-time yields, shorter cycle times and less waste generation. Furthermore, by coupling steps together unfavorable equilibria can be driven toward product (see later).

Notwithstanding the considerable benefits, catalytic cascade processes are fraught with several problems. Different catalysts, e.g., combinations of biocatalysts and chemocatalysts, are often incompatible. The rates may be very different and the optimum conditions for each catalyst may differ considerably and recycling of a complex mixture of catalysts will not be simple. It is worth noting, in this context, that biocatalytic processes generally proceed under the same conditions: in water at ambient temperature, pressure, and physiological pH. In the living cell interference between the different biocatalytic steps is circumvented by compartmentalization in/behind membranes. Following Nature’s example, the key to compatibility would appear to be compartmentalization. This could be achieved, for example, by immobilizing two or more (bio)catalysts, thereby avoiding interference between them. We have achieved this, by immobilizing two or more enzymes in “combi CLEAs,” by coprecipitation and cross-linking. For example, we have successfully prepared combi CLEAs containing catalase in combination with glucose oxidase or galactose oxidase, respectively (R. Schoevaart, manuscript in preparation). The catalase serves to catalyze the rapid degradation of the hydrogen peroxide formed in the aerobic oxidation of glucose and galactose, respectively, catalyzed by these enzymes, thus suppressing deactivation of the enzyme by the hydrogen peroxide.

We [45] have recently used a combi CLEA containing an *S*-selective oxynitrilase (from *Manihot esculenta*) and a nonselective nitrilase, in diisopropyl ether/water (9:1) at pH 5.5, 1 h for the one-pot conversion of benzaldehyde to *S*-mandelic acid (Figure 12.4), in high yield and enantioselectivity.

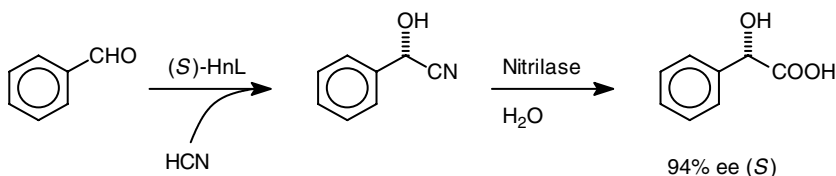


FIGURE 12.4 One-pot conversion of benzaldehyde to *S*-mandelic acid with a combi CLEA.

The enantioselectivity is provided by the oxynitrilase and *in situ* conversion by the nitrilase serves to drive the equilibrium of the first step toward product. In principle, this could also be achieved by using an *S*-selective nitrilase in combination with nonenzymatic hydrocyanation (as we have previously shown with an *R*-nitrilase) but, unfortunately, there are no nitrilases that exhibit *S*-selectivity with mandelonitriles. We also demonstrated that the combi CLEA was more effective than a mixture of the two separate CLEAs.

12.10 CONCLUSIONS AND PROSPECTS

The CLEA technology has many advantages in the context of industrial applications. The method is exquisitely simple and amenable to rapid optimization, which translates to low costs and short time-to-market. It is applicable to a wide variety of enzymes, including crude preparations, affording stable, recyclable catalysts with high retention of activity.

In contrast to CLECs, there is no need for the enzyme to be available in crystalline form and the technique can be applicable to the preparation of combi CLEAs containing two or more enzymes. Synthesis of CLEAs in the presence of additives, such as crown ethers or surfactants, provides the possibility of “locking” the immobilized enzyme in a more favorable conformation, resulting in increased activity and/or (enantio)selectivities. We believe that CLEAs will, in the future, be widely applied in industrial biotransformations and other areas requiring immobilized enzymes.

REFERENCES

1. Sheldon, R.A., Atom efficiency and catalysis in organic synthesis, *Pure Appl. Chem.*, 72, 1233–1246, 2000.
2. Straathof, A.J.J., Panke, S., and Schmid, A., The production of fine chemicals by biotransformations, *Curr. Opin. Biotechnol.*, 13, 548–556, 2002.
3. Patel, R.N., Biocatalytic synthesis of intermediates for the synthesis of chiral drug substances, *Curr. Opin. Biotechnol.*, 12, 587–604, 2001.
4. Patel, R.N., Microbial/enzymatic synthesis of chiral intermediates for pharmaceuticals, *Enzyme. Microb. Technol.*, 31, 804–826, 2002.
5. Liese, A. and Vilella, F.M., Production of fine chemicals using biocatalysis, *Curr. Opin. Biotechnol.*, 10, 595–603, 1999.
6. Schmid, A., Dordick, J.S., Hauer, B., et al., Industrial biocatalysis today and tomorrow, *Nature*, 409, 258–268, 2001.
7. Liese, A., Seelbach, K., and Wandrey, C., *Industrial Biotransformations*, Wiley-VCH, Weinheim, 2000.
8. Thomas, S.M., DiCosimo, R., and Nagarajan, V., Biocatalysis: applications and potentials for the chemical industry, *TIBTECH*, 20, 238–242, 2002.
9. White Biotechnology: Gateway to a More Sustainable Future, EuropaBio Report, 2003, www.europabio.org.
10. Schoemaker, H.E., Mink, D., and Wubbolts, M.G., Dispelling the myths—biocatalysis in industrial synthesis, *Science*, 299, 1694–1697, 2003.
11. Schmidt, A., Hollmann, F., Park, J.B., et al., The use of enzymes in the chemical industry in Europe, *Curr. Opin. Biotechnol.*, 13, 359–366, 2002.
12. Laumen, K., Kittelmann, M., and Ghisalba, O., Chemo-enzymatic approaches for the creation of novel chiral building blocks and reagents for pharmaceutical applications, *J. Mol. Catal., B Enzym.*, 19–20, 55–66, 2002.
13. Shaw, N.M., Robins, K.T., and Kiener, A., Lonza: 20 years of biotransformations, *Adv. Synth. Catal.*, 345, 425–435, 2003.
14. Zaks, A., Industrial biocatalysis, *Curr. Opin. Chem. Biol.*, 5, 130–136, 2001.

15. Yazbek, D.R., Martinez, C.A., Hu, A., et al., Challenges in the development of an efficient enzymatic process in the pharmaceutical industry, *Tetrahedron Asymmetry*, 15, 2757–2763, 2004.
16. Huisman, G.W. and Gray, D., Towards novel processes for the fine-chemical and pharmaceutical industry, *Curr. Opin. Biotechnol.*, 13, 352–358, 2002.
17. Stewart, J.D., Dehydrogenases and transaminases in asymmetric synthesis, *Curr. Opin. Chem. Biol.*, 5, 120–129, 2001.
18. DeSantis, G., Burk, M.J., et al., An enzyme library approach to biocatalysis: development of nitrilases for enantioselective production of carboxylic acid derivatives, *J. Am. Chem. Soc.*, 124, 9024–9025, 2002.
19. Powell, K.A., Ramer, S.W., del Cardayré, S.B., et al., Directed evolution and biocatalysis, *Angew. Chem. Int. Ed. Engl.*, 40, 3948–3959, 2001.
20. Reetz, M.T. and Jaeger, K.-E., Enantioselective enzymes for organic synthesis created by directed evolution, *Chem. Eur. J.*, 6, 407–412, 2000.
21. Minshull, J. and Stemmer, W.P.C., Protein evolution by molecular breeding, *Curr. Opin. Chem. Biol.*, 3, 284–290, 1999.
22. May, O., Nguyen, P.T., and Arnold, F.H., Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine, *Nature Biotechnol.*, 18, 317–320, 2000.
23. Cao, L., van Langen, L.M., and Sheldon, R.A., Immobilised enzymes: carrier-bound or carrier-free, *Curr. Opin. Biotechnol.*, 14, 387–394, 2003.
24. Doscher, M.S. and Richards, F.M., The activity of an enzyme in the crystalline state: ribonuclease S, *J. Biol. Chem.*, 238, 2399–2406, 1963.
25. Quiocho, F.A. and Richards, F.M., Intermolecular cross-linking of a protein in the crystalline state: carboxypeptidase A, *Proc. Natl. Acad. Soc. USA*, 114, 7314–7316, 1992.
26. St. Clair, N.L. and Navia, M.A., Cross-linked enzyme crystals as robust biocatalysts, *J. Am. Chem. Soc.*, 114, 7314–7316, 1992.
27. Margolin, A.L., Novel crystalline catalysts, *Tibtech.*, 14, 223–230, 1996.
28. Margolin, A.L. and Navia, M.A., Protein crystals as novel catalytic materials. *Angew. Chem. Int. Ed. Engl.*, 40, 2204–2222, 2001.
29. Lalonde, J., Practical catalysis with enzyme crystals, *Chemtech.*, 27(2), 38–45, 1997.
30. Haring, D. and Schreier, P., Cross-linked enzyme crystals, *Curr. Opin. Biotechnol.*, 3, 35–38, 1999.
31. Brown, D.L. and Glatz, C.E., Aggregate breakage in protein precipitation, *Chem. Eng. Sci.*, 47, 1831–1839, 1986.
32. Cao, L., van Rantwijk, F., and Sheldon, R.A., Cross-linked enzyme aggregates: a simple and effective method for the immobilization of penicillin acylase, *Org. Lett.*, 2, 1361–1364, 2000.
33. Cao, L., van Langen, L.M., van Rantwijk, F., et al., Cross-linked aggregates of penicillin acylase: robust catalysts for the synthesis of β -lactam antibiotics, *J. Mol. Catal., B Enzym.*, 11, 665–670, 2001.
34. Wegman, M.A., Janssen, M.H.A., van Rantwijk, F., et al., Cross-linked aggregates of penicillin acylase: robust catalysts for the synthesis of β -lactam antibiotics, *Adv. Synth. Catal.*, 343, 559–576, 2001.
35. van Langen, L.M., Oosthoek, N.H.P., van Rantwijk, F., et al., Penicillin acylase catalysed synthesis of ampicillin in hydrophilic organic solvents, *Adv. Synth. Catal.*, 345, 797–801, 2003.
36. Wilson, L., Illanes, A., Abián, O., et al., Co-aggregation of penicillin G acylase and polyionic polymers: an easy methodology to prepare enzyme biocatalysts stable in organic media, *Biomacromolecules*, 5, 852–857, 2004.
37. Wilson, L., Illanes, A., Pessela, B., et al., Encapsulation of crosslinked penicillin G acylase aggregates in lentikats: evaluation of a novel biocatalyst in organic media, *Biotechnol. Bioeng.*, 86, 558–562, 2004.
38. Lopez-Serrano, P., Cao, L., van Rantwijk, F., et al., Cross-linked enzyme aggregates with enhanced activity: application to lipases, *Biotechnol. Lett.*, 24, 1379–1383, 2002.
39. Theil, F., Enhancement of selectivity and reactivity of lipases by additives, *Tetrahedron*, 56, 2905–2909, 2000.
40. Mateo, C., Palomo, J.M., van Langen, L.M., et al., A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates, *Biotechnol. Bioeng.*, 86, 273–276, 2004.

41. Schoevaart, R., Wolbers, M.W., Golubovic, M., et al., Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs), *Biotechnol. Bioeng.*, 87, 754–762, 2004.
42. Wilson, L., Betancor, L., Fernandez-Lorente, G., et al., Cross-linked aggregates of multimeric enzymes: a simple and efficient methodology to stabilize their quaternary structure, *Biomacromolecules*, 5, 814–817, 2004.
43. Hilal, N., Nigmatullin, R., and Alpatova, A., Immobilization of cross-linked lipase aggregates within microporous polymeric membranes, *J. Memb. Sci.*, 238, 131–141, 2004.
44. Bruggink, A., Schoevaart, R., and Kieboom, T., Concepts of nature in organic synthesis: cascade catalysis and multistep conversions in concert, *Org. Proc. Res. Dev.*, 7, 622–640, 2003.
45. Mateo, C. Chmura, A., Rustler, S., van Rantwijk, F., Stolz, A., Sheldon, R.A., Synthesis of enantiomerically pure (S)-mandelic acid using an oxynitrilase-nitrilase bienzymatic cascade: a nitrilase surprisingly shows nitrile hydratase activity, *Tetrahedron: Asymmetry*, 17, 320–323, 2006.

13 Biotechnological Applications of Aldolases

Wolf-Dieter Fessner and Stefan Jennewein

CONTENTS

13.1	Introduction	363
13.2	Mechanistic Distinctions	364
13.3	Dihydroxyacetone Phosphate-Dependent Aldolases	365
13.4	Pyruvate- (and Phosphoenolpyruvate-) Dependent Lyases	377
13.5	2-Deoxyribose 5-Phosphate Aldolase	382
13.6	Glycine-Dependent Aldolases	387
13.7	Transketolase	388
13.8	Perspectives	391
	References	392

13.1 INTRODUCTION

The demand for enantiomerically pure compounds, in particular for pharmaceutical application, has steered immense interest in the industrial asymmetric synthesis of chiral products and intermediates [1]. As an alternative to classical chemical methodology, enzyme-catalyzed reactions were used early on in chemical research and production, and are now gaining increasing attention [2,3]. Indeed, processes using hydrolytic enzymes for biocatalytic resolution of racemates constitute the majority of current applications on industrial scale [4]. However, standard resolution approaches to enantiopure compounds can only achieve a maximum molar yield of 50%.

Undoubtedly, the direct stereoselective synthesis of asymmetric molecules from prochiral precursors using chiral catalysts represents an attractive alternative. In particular, stereoselective carbon-carbon bond forming reactions are among the most useful synthetic methods in asymmetric synthesis because this allows the simultaneous creation of up to two adjacent stereocenters [5]. Several classes of enzymes are able to catalyze stereoselective C-C bond formation reactions (lyases) such as aldolases [6], oxynitrilases [7], or thiamine diphosphate-dependent enzymes [8]. Especially, the high stereospecificity of aldolases in C-C bond forming reactions gives them substantial utility as synthetic biocatalysts, and makes them an environmentally benign alternative to chiral transition metal catalysis for the asymmetric aldol reaction [9,10]. In fact, the industrial process based on yeast pyruvate decarboxylase for the production of (*R*)-phenylacetyl carbinol, a precursor to (–)-ephedrine, is still utilized since its invention 70 years ago [11].

In vivo, aldolases are involved in the metabolism of amino acids and carbohydrates, compounds that are important *per se*, or as chiral building blocks for more complex pharmaceuticals. Although this may seem to limit the scope of potential practical applications,

such enzymes open new windows of synthetic opportunity because these polyfunctional classes of compounds are difficult to prepare and handle by conventional organic chemical methods and mandate the laborious manipulation of protective groups. Due to the high levels of selectivity offered by enzymes under their mild operating conditions that are compatible with most functional groups, biocatalytic conversions can usually be performed on nonderivatized substrates, thus making tedious and costly protecting group manipulations superfluous [12].

13.2 MECHANISTIC DISTINCTIONS

Aldolases achieve the activation of an aldol donor substrate by two mechanistically different pathways (Figure 13.1). Class I aldolases (a) bind their substrates covalently at an active site lysine residue through an imine–enamine intermediate to initiate C–C bond formation or cleavage [13]. In contrast, class II aldolases (b) utilize transition metal ions as a Lewis acid cofactor, which facilitates the stereospecific deprotonation of the donor by bidentate coordination to stabilize the enediolate nucleophile (Figure 13.1) [14]. This effect is usually achieved by means of a tightly bound Zn^{2+} ion. The mechanistic models for both classes of aldolases have been substantiated by several crystal structures of enzymes liganded by substrate or inhibitor that altogether provide a detailed insight into the catalytic function and the individual contribution of active site residues to the stereochemically determining event [6].

Apart from their mechanistic distinction, aldolases can be subdivided into certain families depending on their nucleophilic substrate: (1) dihydroxyacetone phosphate

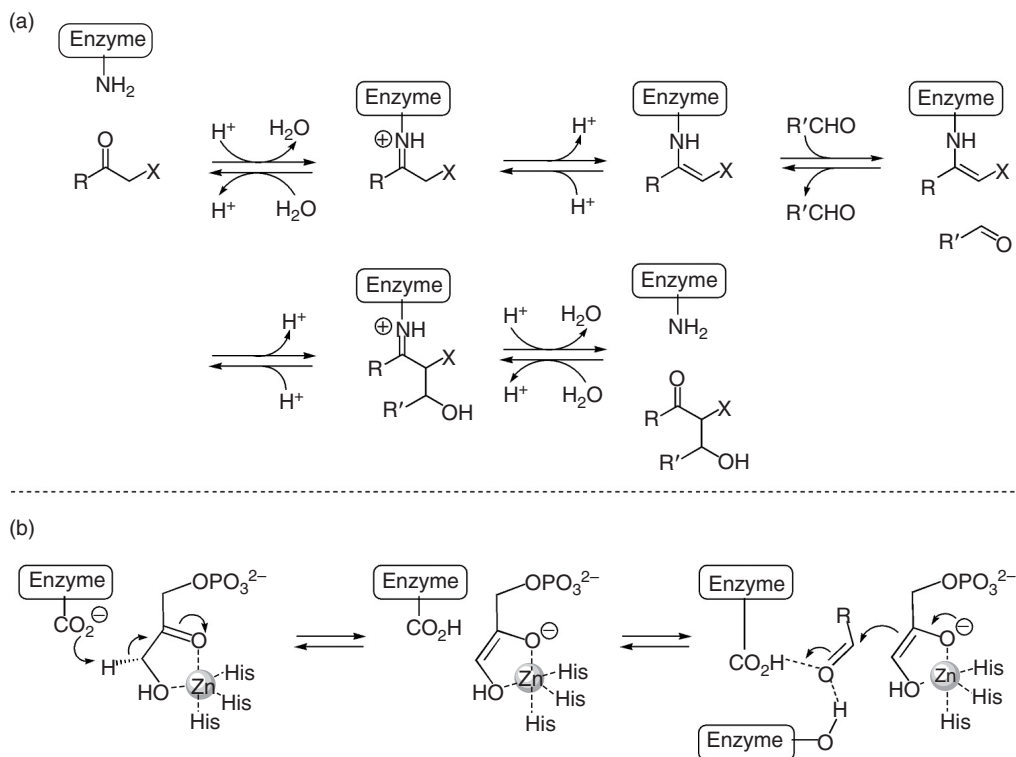


FIGURE 13.1 Schematic mechanism for aldolases of class I (a) and class II (b).

(DHAP)-dependent aldolases, (2) pyruvate- (and phosphoenolpyruvate-)dependent lyases, (3) aldehyde-dependent aldolases, and (4) glycine-dependent enzymes (Figure 13.2). Members of the first two families all add 3-carbon ketone fragments to the carbonyl group of an aldehyde, yielding 1,3,4-trihydroxylated methyl ketone derivatives or 3-deoxy-2-oxoacids, respectively. The latter two families use the carbon fragments acetaldehyde and glycine, respectively, as the nucleophilic component. Today, many useful synthetic applications have been demonstrated for all classes of aldolases and for related C–C bond forming enzymes. Due to the nature of the different substrates used and compound classes generated, the individual aldolase families will be treated separately with regard to their synthetic potential.

Because of the mechanistic complexity inherent to the C–C bond forming process, which requires the stereocontrolled binding and dipolar activation of two substrates, and because of some unique properties of the individual enzymes, still only a limited number of industrial lead processes have been successfully developed. Thus, this chapter will also deal with interesting synthetic examples, and prominent operating technology, to demonstrate preparative opportunities and to stimulate imagination toward future biotechnology developments.

13.3 DIHYDROXYACETONE PHOSPHATE-DEPENDENT ALDOLASES

While all aldolases typically represent catabolic enzymes, DHAP-dependent aldolases are involved in the degradation of phosphorylated ketosugars. A particular advantage for synthetic applications is the fact that nature has evolved a full set of four stereochemically complementary enzymes, which are termed as D-fructose 1,6-bisphosphate aldolase (FruA), D-tagatose 1,6-bisphosphate aldolase (TagA), L-fucose 1-phosphate aldolase (FucA), and L-rhamnulose 1-phosphate aldolase (RhuA), based on their capacity to cleave the corresponding diastereoisomeric ketose 1-phosphates **6–9** in a retroaldol manner (Figure 13.3). In the direction of synthesis, this formally allows the preparation of any one of the four possible diastereomeric aldol adducts by simply choosing the appropriate enzyme and

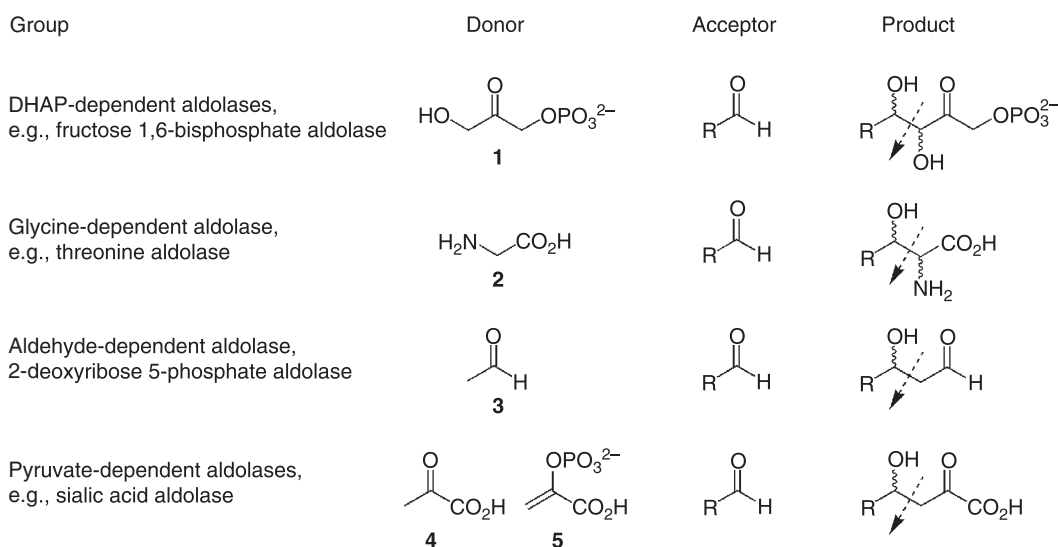


FIGURE 13.2 Subgrouping of aldolases according to donor type.

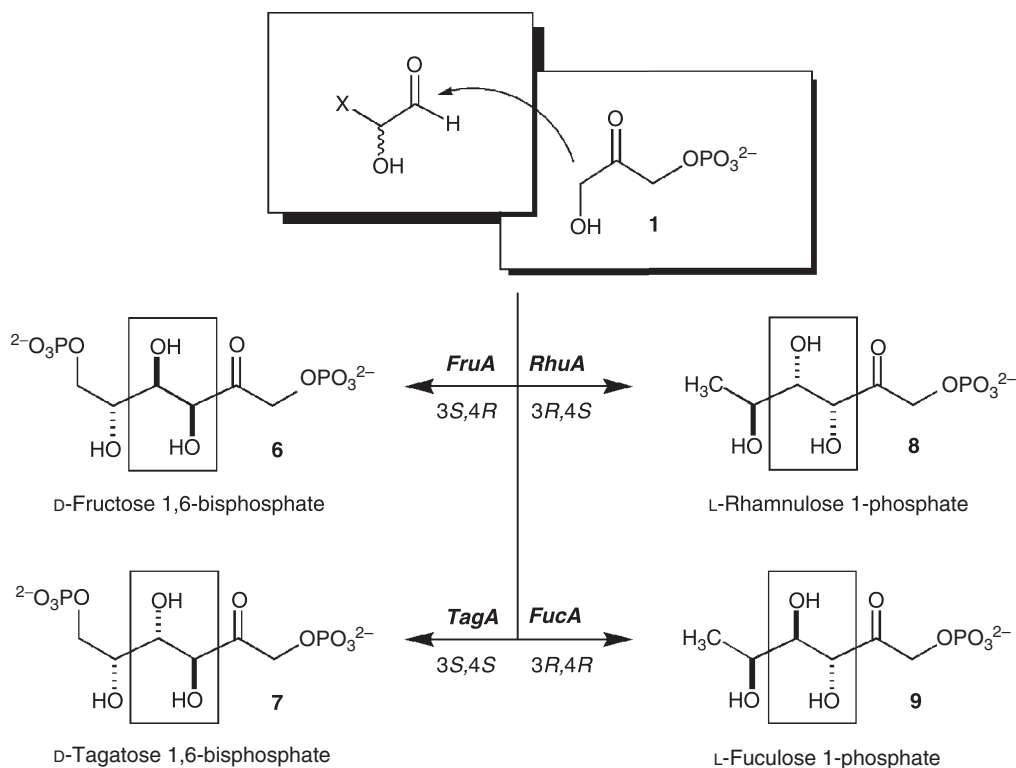


FIGURE 13.3 Stereochemically complementary set of dihydroxyacetone phosphate-dependent aldolases.

starting materials for a full control over constitution and absolute configuration of the desired product [9].

All four members of the DHAP-dependent aldolase family are represented by Zn^{2+} -dependent types (class II), and Schiff-base-forming types (class I) are known for FruA and TagA. As a rule of thumb, metalloaldolases are more stable than their Schiff-base-forming relatives. In the presence of low concentrations of their metal cofactor, they possess half-lives of several weeks or even months in solution, as compared to only a few days [15–17], and can tolerate the presence of significant portions of organic cosolvents ($\geq 30\%$), such as DMSO, DMF, and ethanol [17]. Notable exceptions are the class I FruA members found in *Staphylococcus* strains, which show unusual thermal and process stability [18].

The FruA, and in particular the class I rabbit muscle aldolase enzyme (RAMA), is the most extensively studied DHAP-dependent aldolase [15]. *In vivo*, FruA catalyzes the pivotal reaction of glycolysis, which is the reversible cleavage of D-fructose 1,6-bisphosphate (**6**) to D-glyceraldehyde 3-phosphate and DHAP (**1**). The equilibrium constant of the reaction of 10^4 M^{-1} strongly favors the synthesis direction [19]. Similarly, TagA is involved in the catabolism of D-galacto-configured carbohydrates [20]. Both RhuA and FucA are derived from closely parallel microbial degradation pathways of L-rhamnose and L-fucose, respectively, where they cleave the corresponding ketose 1-phosphate (**8,9**) into **1** and L-lactaldehyde [21–23]. The latter two aldolases have been studied for production at larger volumetric scale [24,25].

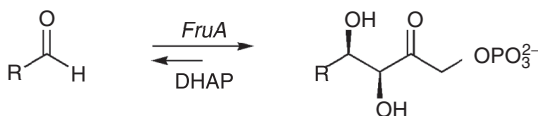
DHAP-dependent aldolases have proved to be exceptionally powerful tools for asymmetric synthesis, particularly for the stereocontrolled synthesis of polyoxygenated compounds due to their relaxed acceptor specificity for aldehyde substrates and a generally high level of

stereocontrol. Class I FruA enzymes show a very broad tolerance for structurally diverse aldehyde substrates, and hundreds of aldehydes have so far been tested successfully to function as acceptor substrate in enzymatic assays or preparative experiments [26]. Table 13.1 lists a small compilation of aldehydes tested as replacement for D-glyceraldehyde 3-phosphate and the corresponding aldol products. Similar to FruA, also FucA and RhuA enzymes show broad acceptance of variously substituted aldehydes, compiled in Table 13.2, with conversion rates useful for synthetic applications [17]. Conversely, microbial FruA enzymes (class II) from *Escherichia coli* and yeast display high substrate specificity for phosphorylated substrates [9].

In direction of synthesis, stereospecificity for the natural configuration is somewhat substrate-dependent, in that small aliphatic aldehydes can give rise to a certain fraction of the diastereomer having an opposite configuration at C-4 [9,17]. In general, stereochemical fidelity is usually higher, and diastereospecific results are observed more often with FruA and FucA than with RhuA. While class I TagA enzymes examined from *Staphylococcus* and *Streptococcus* sp. have no stereochemical preference with regard to D-tagatose or D-fructose configuration [27], the class II enzyme from *E. coli* is highly stereoselective for its natural substrate in both cleavage and synthesis direction [28–30]. The aldolase also accepts a range of unphosphorylated aldehydes as electrophilic substrates, but unfortunately produces only mixtures of diastereomers [28].

In addition to the high-stereoselective formation of the C–C bond, both RhuA and FucA show strong kinetic preference for L-configured enantiomers of 2-hydroxyaldehydes

TABLE 13.1
Substrate Tolerance of Fructose 1,6-Bisphosphate Aldolase



R	Relation Rate (%)	Yield (%)
D-CHOH-CH ₂ OPO ₃ ²⁻	100	95
H	105	—
CH ₃	120	—
CH ₂ Cl	340	50
CH ₂ -CH ₃	105	73
CH ₂ -CH ₂ -COOH	—	81
CH ₂ OCH ₂ C ₆ H ₅	25	75
D-CH(OCH ₃)-CH ₂ OH	22	56
CH ₂ OH	33	84
D-CHOH-CH ₃	10	87
L-CHOH-CH ₃	10	80
DL-CHOH-C ₂ H ₅	10	82
CH ₂ -CH ₂ OH	—	83
CH ₂ -C(CH ₃) ₂ OH	—	50
DL-CHOH-CH ₂ F	—	95
DL-CHOH-CH ₂ Cl	—	90
DL-CHOH-CH ₂ -CH=CH ₂	—	85

TABLE 13.2

Substrates Accepted by L-Rhamnulose 1-Phosphate and L-Fuculose 1-Phosphate Aldolases

R	RhuA Rel. Rate (%)	Selectivity <i>threo/erythro</i>	Yield (%)	FucA Rel. rate (%)	Selectivity <i>threo/erythro</i>	Yield (%)
L-CH ₂ OH-CH ₃	100	>97:3	95	100	<3:97	83
CH ₂ OH	43	>97:3	82	38	<3:97	85
D-CHOH-CH ₂ OH	42	>97:3	84	28	<3:97	82
L-CHOH-CH ₂ OH	41	>97:3	91	17	<3:97	86
CH ₂ -CH ₂ OH	29	>97:3	73	11	<3:97	78
CHOH-CH ₂ OCH ₃	—	>97:3	77	—	<3:97	83
CHOH-CH ₂ N ₃	—	>97:3	97	—	<3:97	80
CHOH-CH ₂ F	—	>97:3	95	—	<3:97	86
H	22	—	81	44	—	73
CH ₃	32	69:31	84	14	5:95	54
CH(CH ₃) ₂	22	97:3	88	20	30:70	58

(Figure 13.4), which facilitates an effective racemate resolution [31,32]. This feature enables the concurrent determination of three contiguous chiral centers in the final products (e.g., **10**, **12**) starting from readily accessible racemic aldehyde substrates. Enantiomer discrimination by rabbit muscle FruA is limited to its natural substrate (20:1 preference for D-glyceraldehyde 3-phosphate over the L-antipode), but fails for nonphosphorylated aldehydes [15,33].

Due to the freely reversible nature of the aldol reactions, a high level of enantiomeric differentiation can be gained alternatively by thermodynamic control under fully equilibrating conditions, when diastereomeric products adopt cyclic half acetals that have distinct relative stability (Figure 13.5). Using racemic 2- or 3-hydroxylated aldehyde substrates, 3.5-fold and 33-fold discrimination for the more stable *trans* and all-equatorial isomer has been reported, respectively [15,34,35]. This thermodynamic product control (e.g., **17/23/27** over **18/24/26**) was utilized in the preparation of novel 4,6-deoxy sugars such as 4-deoxy-L-fucose (**19**) from racemic 3-hydroxy-butanal (**14**) [9], and in the synthesis of unsaturated higher sugars starting from racemic unsaturated aldehydes [34,35].

Owing to their metabolic function, early utilization of aldolases concerned the preparative synthesis of ketoses (e.g., **30**) through the corresponding ketose 1-phosphates, notably also of isotopically labeled derivatives ¹³C-enriched at defined positions [36–38]. Taking advantage of the kinetic selectivity of RhuA for L-configured 2-hydroxyaldehydes, nonnatural L-fructose (**31**) is accessible starting from DHAP to racemic glyceraldehyde (Figure 13.6) [39].

The scope and synthetic usefulness of FruA for the synthesis of novel monosaccharides and related molecules are further illustrated by the preparation of various unusual products: including branched-chain (**34**) and spiroannulated sugars (**37,38**) [40], natural product analogs such as nucleoside (**35**) [41] or oligosaccharide mimetics (**39**) [42], or a perfluoroalkylated fructose surfactant (**36**) [43], each synthesized by FruA from the corresponding aldehyde precursors (Figure 13.6).

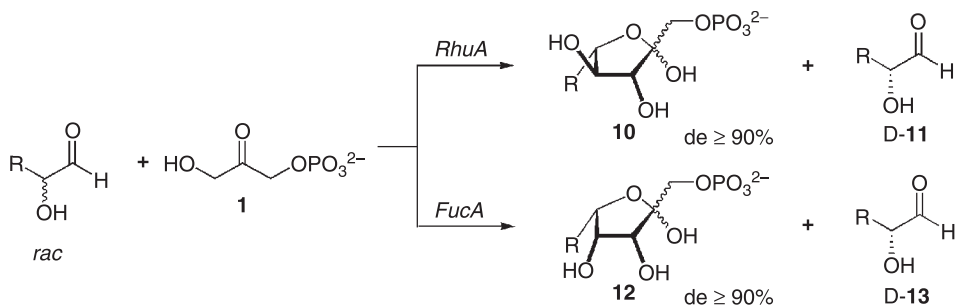


FIGURE 13.4 Kinetic enantioselectivity of class II DHAP-dependent aldolases useful for racemic resolution of 2- hydroxyaldehydes.

Complex 8- and 9-carbon monosaccharide derivatives, representing sialic acid or KDO analogs, could be obtained from pentose and hexose monophosphates by stereospecific chain extension using FruA from rabbit muscle (Figure 13.7) [44]. This approach provides a convenient route to novel α,ω -phosphorylated high-carbon sugars (e.g., **41**, **43**), which are difficult to obtain from either natural sources or chemical synthesis.

Twofold aldolase-catalyzed chain elongation of α,ω -dialdehydes (“tandem” aldolization) has been developed into an efficient method for the generation of high-carbon sugars (e.g., **45**)

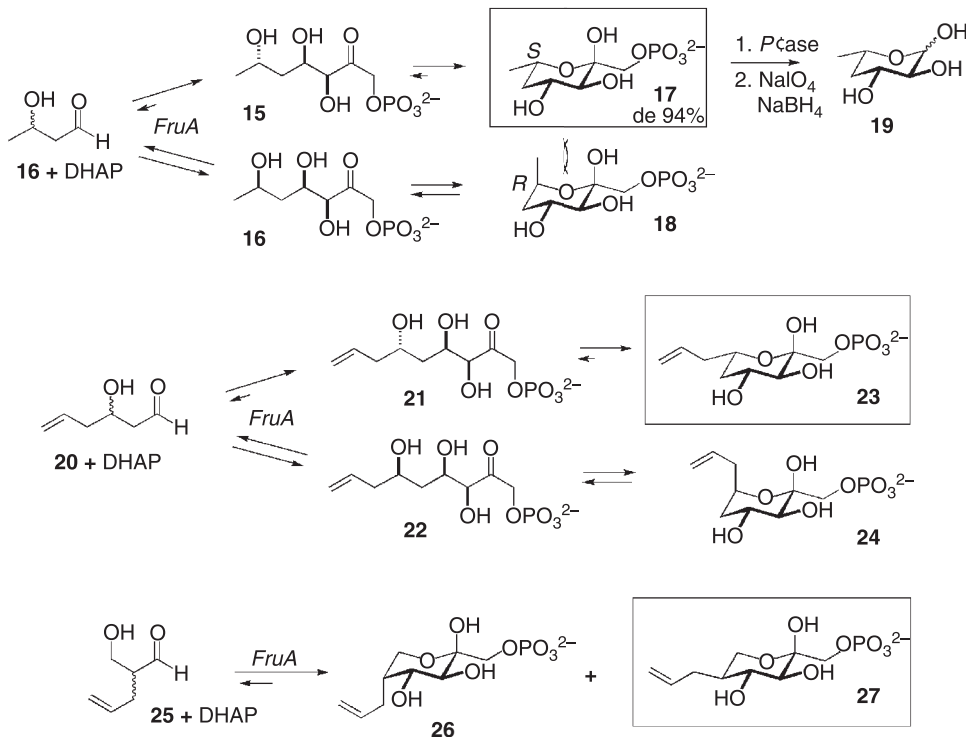


FIGURE 13.5 Thermodynamically controlled diastereoselectivity in FruA-catalyzed aldol additions under equilibrating conditions.

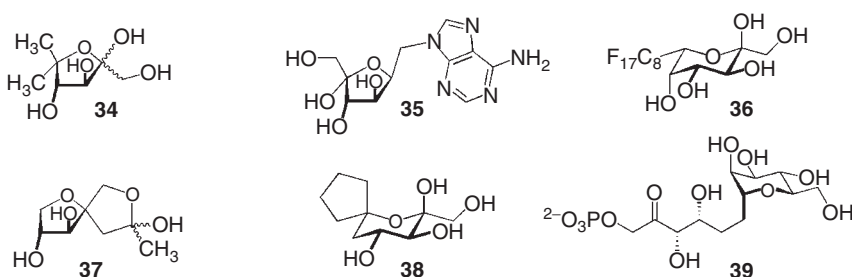
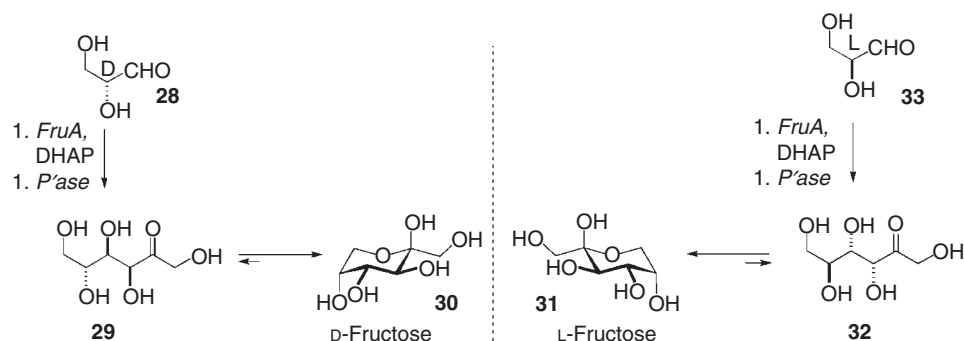


FIGURE 13.6 Enzyme-catalyzed asymmetric synthesis of D- and L-fructose by stereocomplementary aldolases, and selected examples of carbohydrate-related products accessible by enzymatic aldolization.

by simple one-pot operations (Figure 13.7) [45]. The overall specific substitution pattern in the carbon-linked disaccharide mimetics is deliberately addressable by the relative hydroxyl configuration in the starting material and choice of the aldolase. Single diastereomers favoring *trans* or equatorial connectivity such as that present in **45** may be obtained in good overall yield even from racemic precursors, if the tandem aldolizations are conducted under thermodynamic control (cf., Figure 13.5). Highly complex structures like anulated (**47**) and spirocyclic (**49**) carbohydrate mimics may be obtained from appropriately customized precursors (Figure 13.7) [46].

Reaction products of DHAP aldolases are typically ketoses; however, isomeric aldoses are often more valuable. The utility of products from FruA, FucA, and RhuA catalyzed reactions is further extended due to the existence of corresponding ketol isomerases that convert the ketose products to the corresponding aldose isomers. Stereochemically complementary L-rhamnose (RhaI; EC 5.3.1.14) and L-fucose isomerases (FucI; EC 5.3.1.3) have been found to be specific for a (3*R*)-OH configured sugar product, but are tolerant to modifications in stereochemistry or substitution pattern at other positions [9,31,47]. This strategy has been clearly illustrated by the synthesis of new L-fucose analogs (**55**) and other L-configured aldohexoses using different enzyme combinations (Figure 13.8) [47,48]. Similar results have been realized by utilizing a glucose isomerase (GlcI; EC 5.3.1.5), which is an industrially important enzyme for the isomerization of D-glucose to D-fructose but has a more narrow specificity. The latter enzyme also accepts derivatives and analogs of D-fructose and has been used in combined enzymatic syntheses, particularly of 6-modified D-glucose derivatives [49].

Access to aldoses can be also achieved by an “inversion strategy” (Figure 13.8), which utilizes a monoprotected dialdehyde (e.g., **56**) for aldolization and, after stereoselective ketone reduction, provides free aldose (**58**) upon liberation of the masked aldehyde function [50].

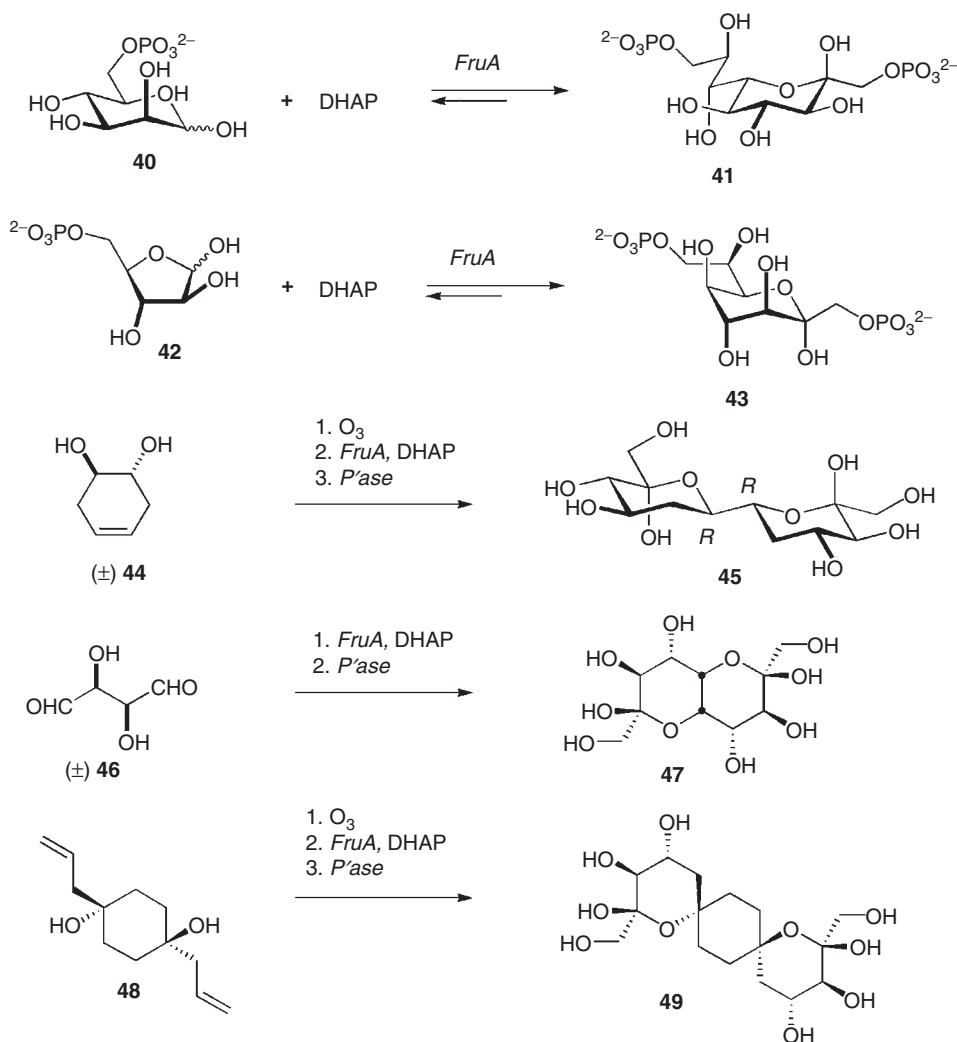


FIGURE 13.7 FruA-catalyzed enzymatic synthesis of α,ω -bisphosphate esters of higher carbon sugars, and bidirectional chain extension of dialdehyde substrates yielding potential disaccharide and oligosaccharide mimetics.

Stemming from the lessons learned in the synthesis of rare and novel monosaccharides, a major opportunity has been developed toward the preparation of “aza sugars,” which represent powerful glycosidase inhibitors or show potent antiviral activity [51]. This rather flexible synthetic strategy consists of an aldol addition to an *N*-functionalized aldehyde, which usually contains an azide group, followed by hydrogenolytic intramolecular reductive amination of the keto functionality (including prior azide to amine reduction) to close an *N*-heterocyclic ring structure stereoselectively [52]. Prominent examples concern the FruA-catalyzed synthesis of deoxynojirimycin (**62**) and deoxymannojirimycin (**63**) from 3-azido-2-hydroxypropanal (**59**) (Figure 13.9) [16,53–56], which represent natural products found in plants or microorganisms. It is important to note that the stereodivergent preparation of a large variety of diastereomeric aza sugars of the nojirimycin type from any single azido aldehyde (such as **59**) can be achieved by choosing from the DHAP aldolases having different stereospecificity [9].

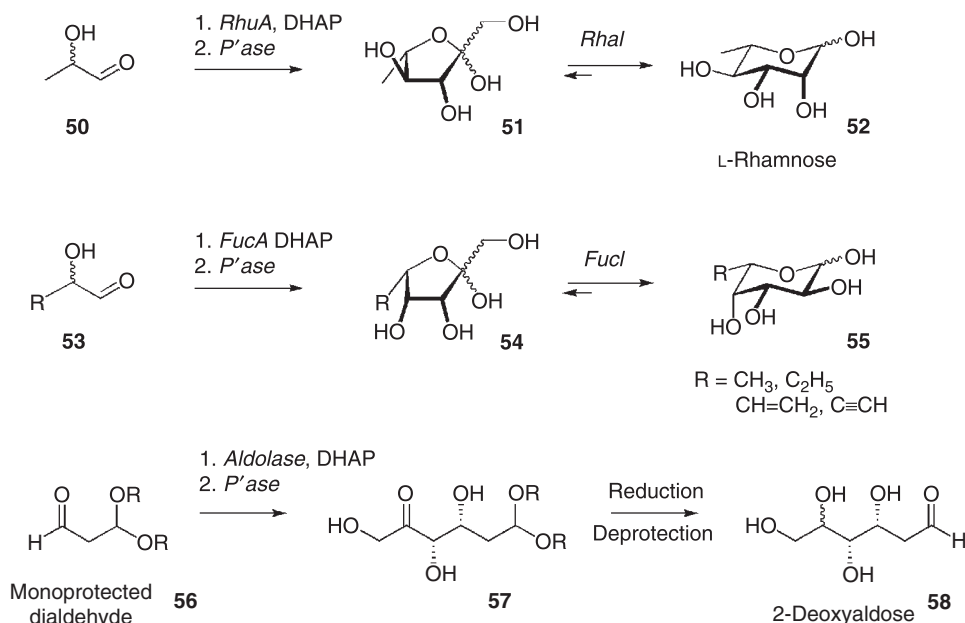


FIGURE 13.8 Enzymatic synthesis of L-rhamnose and L-fucose as well as hydrophobic analogs by aldolization-ketol isomerization with kinetic resolution of racemic hydroxyaldehydes, and “inversion strategy” for the synthesis of aldoses from monoprotected dialdehydes.

Starting from (*S*)- to (*R*)-3-azido-2-acetamidopropanal in FruA-catalyzed aldol reactions, aza sugar derivatives corresponding to *N*-acetyl-glucosamine and *N*-acetyl-mannosamine have been prepared [57]. Similarly, a FruA-mediated stereospecific DHAP addition to intermediate **64** served as the key step in the chemoenzymatic synthesis of australine (**67**), 3-epiaustraline (**68**), and 7-epialexine (Figure 13.9) [58]. Australine and alexine are naturally occurring pyrrolizidine alkaloids, which are of pharmacological interest due to their potent glycosidase inhibitory and antiviral activities.

Beyond the immediately obvious applications of this type of aldolases to the synthesis of carbohydrates or carbohydrate derived compounds, the enzymes are highly valuable for the determination of asymmetric centers in the construction of stereochemically homogenous fragments of complex noncarbohydrate natural products. An impressive illustration is the FruA-catalyzed chemoenzymatic synthesis of (+)-*exo*-brevicomine (**72**), the aggregation pheromone of the western pine bark beetle *Dendroctonus brevicomis* (Figure 13.10). In two complementary routes, starting from either 5-oxohexanal (**69**) [59] or propanal (**73**) [44], which yields an intermediate **74** identical to that from a corresponding transketolase-catalyzed transformation [60], the only independent two stereocenters of the natural product **72** are induced by the aldolase. Another elegant application employing FruA for the synthesis of a nonsugar molecule represents the synthesis of (–)-syringolide (**79**), a structurally complex tricyclic microbial elicitor in plants (Figure 13.11) [61]. Again, the FruA-catalyzed reaction established the absolute and relative configuration of the vicinal diol defining the only chiral centers that needed to be externally induced in the intermediates **77/78**; the configuration of all other stereocenters seemed to follow by kinetic preference during the subsequent cyclization.

Other remarkable applications of FruA for “noncarbohydrate” synthetic targets involve the stereoselective generation of intermediates (e.g., **81**) for the synthesis of (+)-aspicillin (**82**),

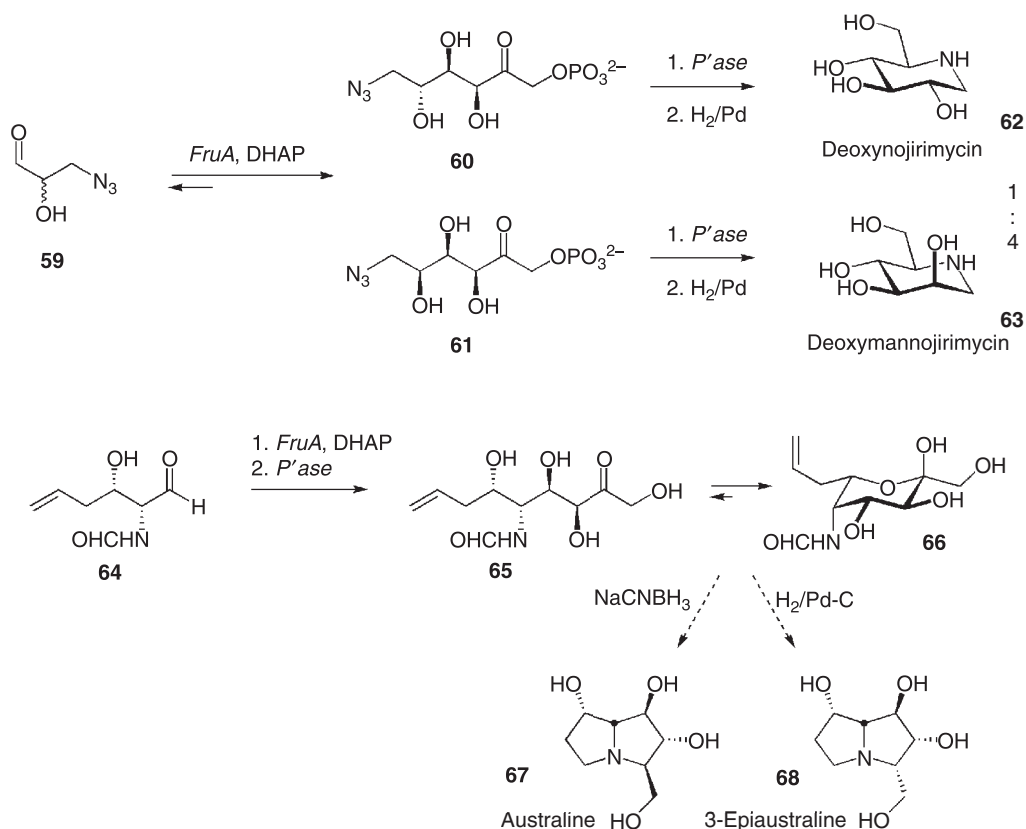


FIGURE 13.9 Chemoenzymatic synthesis of the potent glycosidase inhibitors deoxynojirimycin and deoxymannojirimycin, and of the pyrrolizidine alkaloids australine and 3-epiaustraline.

a lichen macrolactone [62], and of the skipped polyol C9–C16 chain fragment (**84**) of the macrolide antibiotic pentamycin (**85**) (Figure 13.12) [63]. A two-stage enzymatic sequence of arene dihydroxylation, using a naphthalene dioxygenase from *Pseudomonas putida*, followed by RhuA-catalyzed aldolization has been developed for the synthesis of novel analogs of the cytotoxic pancratistatin (**91**) pharmacophore (Figure 13.13) [64]. This strategy converts a naphthalene core (**86**) into a complex hybrid arene–carbohydrate structure (**88**), with simultaneous creation of four contiguous stereocenters, in just three steps.

It seems that all DHAP-dependent aldolases are highly specific for **1** as the nucleophilic component. However, the compound is chemically difficult to prepare, and in addition shows limited stability in solution, particularly at alkaline pH. Several protocols for the chemical synthesis of **1** have been developed [65–72]. However, due to its sensitive nature **1** is generated best enzymatically and consumed *in situ* by enzymatic aldol reaction, which avoids the buildup of high stationary concentrations. Dihydroxyacetone (**96**) can be enzymatically phosphorylated using a glycerol kinase with ATP regeneration [73–75], or by transphosphorylation from phosphatidyl choline using phospholipases [76]. The preparation from glycerol (**93**) by successive phosphorylation/dehydrogenation with an integrated double cofactor recycling scheme has also been developed [77]. A highly practical method for generation of **1** *in situ* is based on the oxidation of L-glycerol phosphate (**91**) catalyzed by a microbial flavine-dependent glycerol phosphate oxidase [78]. The process clearly generates **1** in practically quantitative yield and, due to the insensitivity of the DHAP-dependent

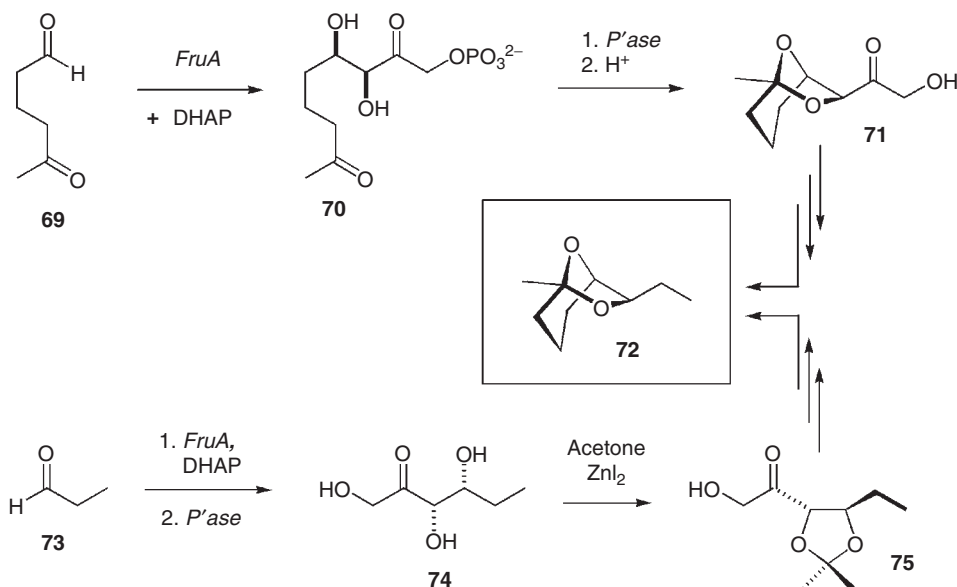


FIGURE 13.10 Complementary, backbone inverting approaches for the asymmetric synthesis of the insect pheromone (+)-*exo*-brevicomin.

aldolases to an oxygenated solution, can be coupled directly to synthetic aldol reactions (Figure 13.14) [79].

Probably the most elegant and convenient method is the *in situ* formation of two equivalents of **1** from commercial fructose 1,6-bisphosphate (**6**) by a combination of FruA and triose phosphate isomerase [15]. This scheme has been extended into a highly integrated “artificial metabolism” for the efficacious *in situ* preparation of **1** from inexpensive feedstock such as glucose and fructose (two equivalents of **1** each), or sucrose (four equivalents of **1**) by a combination of up to seven inexpensive enzymes [40]. When employing the class II FruA of *E. coli* for aldol cleavage of **6**, which displays high substrate specificity for glyceraldehyde-3-phosphate (**95**) and thus is inactive with other added aldehyde substrates, this system can be

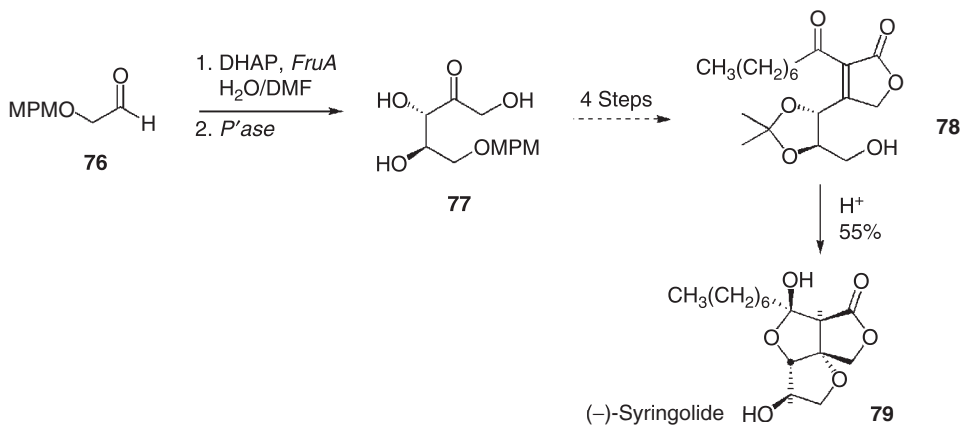


FIGURE 13.11 FruA-based creation of two independent chiral centers in the total synthesis of the complex microbial plant defence elicitor (-)-syringolide.

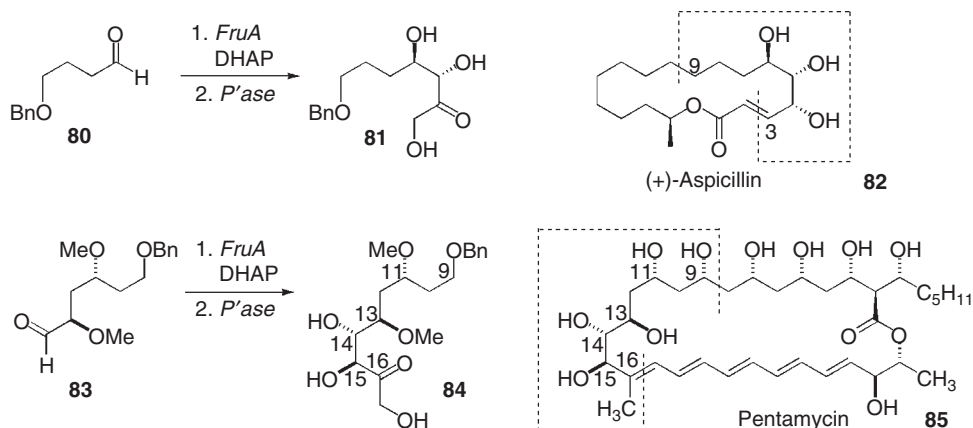


FIGURE 13.12 Stereoselective generation of chiral precursors for the synthesis of the macrolactone (+)-aspicillin and the macrolide antibiotic pentamycin using FruA catalysis.

“metabolically engineered” by adding another aldolase to furnish products having a stereo-configuration different from the starting material **6** (Figure 13.15) [9].

Due to the need of **1** as donor nucleophile, the generated aldol products will contain a phosphate ester moiety. The latter facilitates product isolation, for example, by barium salt precipitation or by use of ion-exchange techniques, but usually is undesired in the final product. The corresponding phosphate free compounds can easily be obtained by enzymatic hydrolysis using alkaline phosphatase at pH 8–9, whereas base labile compounds require acid

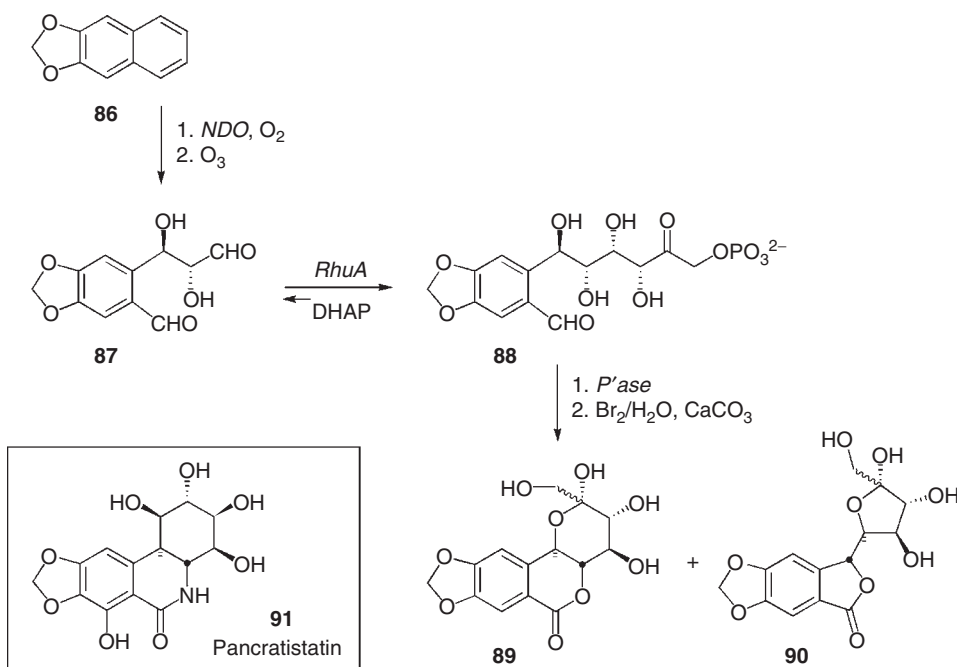


FIGURE 13.13 Chemoenzymatic synthesis of a pancratistatin analog using a RhuA-catalyzed aldolization reaction.

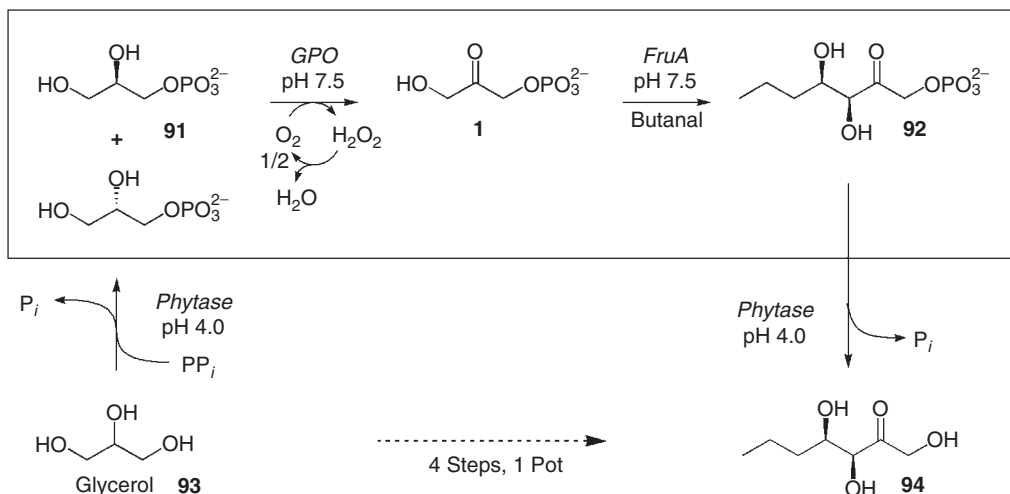


FIGURE 13.14 Enzymatic *in situ* generation of DHAP for stereoselective aldol reactions using DHAP aldolases (box), and extension by pH-controlled, integrated precursor preparation and product liberation.

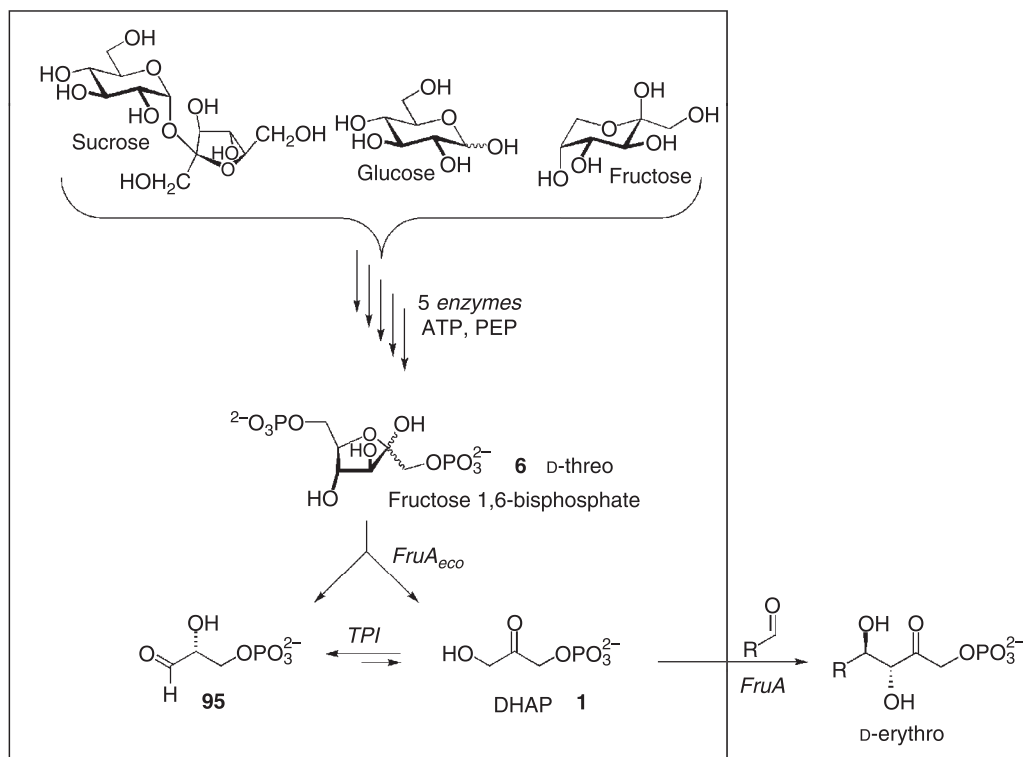


FIGURE 13.15 *In vitro* “artificial metabolism” for the *in situ* preparation of dihydroxyacetone-phosphate along the glycolysis cascade, and utilization for subsequent stereoselective carbon–carbon bond formation using an aldolase with distinct stereoselectivity.

phosphatase treatment. The phosphate moiety also mandates an aqueous medium for solubility reasons and to prevent substrate/product inhibition of the enzymes; for organic substrates having only limited solubility in water, it could be shown that aldolases are compatible with water-in-oil microemulsion systems [80,81]. For industrial applications, the high specificity for **1** and the costs for its preparation severely limit the synthetic usefulness of this enzyme class. A partial solution was offered by coupling the GPO oxidative generation of **1** from **91** with a reversible phosphoryl transfer from inexpensive pyrophosphate to **93** catalyzed by phytase (an acid phosphatase), and staging the phosphorylation–aldolization product dephosphorylation sequence by appropriate pH shifts (Figure 13.14) [82].

Interestingly, dihydroxyacetone (**96**) in the presence of high concentration of inorganic arsenate (≥ 0.5 M) reversibly forms the corresponding monoarsenate ester (**97a**) *in situ*, which can replace **1** in enzyme-catalyzed aldol reactions (Figure 13.16) [83,84]. The method suffers, however, from rather low-reaction rates in addition to the high toxicity of arsenate. Similarly, inorganic vanadate also spontaneously forms the corresponding vanadate ester (**97b**), but the oxidation potential has to be controlled by appropriate buffers to prevent oxidation of **96**. So far, only RhuA of *E. coli* has been shown to accept **97b** in place of **1** for preparative synthesis [9].

The GPO method for generation of **1** can also be used to generate analogs with isosteric replacements of the ester oxygen for sulfur (**98**), nitrogen (**99**), or methylene carbon (**100**) (Figure 13.17) [38], which are substrates of many aldolases of classes I and II [38,85,86]. Thus, sugar phosphonate analogs (e.g., **105**) can be rapidly prepared from **100** that mimic intermediates of carbohydrate metabolism but are stable to hydrolysis. The hydrogenolytic lability of the phosphorothioate analogs (e.g., **102**) makes terminally deoxygenated sugars accessible, as was demonstrated by a stereoselective synthesis of D-olivose (**103**) based on the “inversion strategy” (Figure 13.17) [87].

13.4 PYRUVATE- (AND PHOSPHOENOLPYRUVATE-) DEPENDENT LYASES

Pyruvate-dependent aldolases serve *in vivo* catabolic functions, whereas phosphoenolpyruvate (PEP)-dependent lyases are involved in biosynthetic reactions of 2-keto-3-deoxy acids. The most interesting enzymes for synthetic applications are those involved in the metabolism of sialic acids (e.g., **107**) or KDO (2-keto-3-deoxy-*manno*-octosonate, *ent*-**108**), which represent complex sugars typically found in mammalian or bacterial glycoconjugates [88–90], respectively. Due to thermodynamically often unfavorable equilibrium constants [19], the preparative aldol additions have to be driven by providing excess pyruvate (**4**) to achieve satisfactory

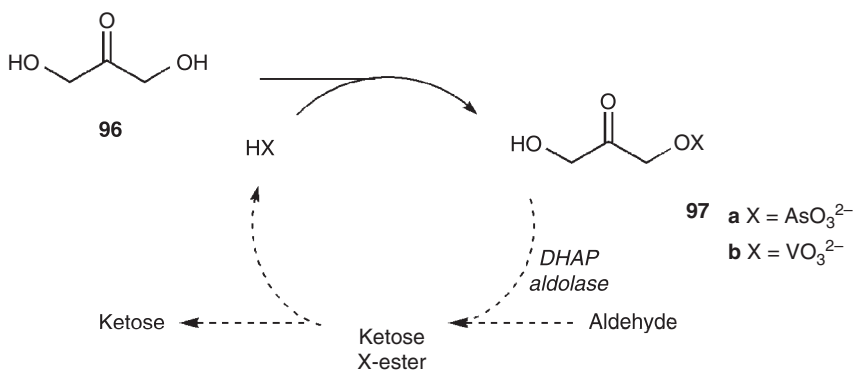


FIGURE 13.16 Spontaneous, reversible formation of arsenate and vanadate analogs of DHAP *in situ* for enzymatic aldol additions.

conversions. In contrast, PEP-dependent lyases (synthetases) use high-energy enol ester (**5**) instead of **4**, which upon C–C bond formation liberates inorganic phosphate, and thus renders the aldol addition essentially irreversible (Figure 13.18). Although attractive from a synthetic point of view, PEP-dependent lyases have not yet been extensively studied for preparative applications [91].

Most extensively studied enzyme in the field of pyruvate-dependent lyases is the *N*-acetylneuraminic acid aldolase (NeuA), also referred to as sialic acid aldolase [9]. NeuA found in both bacteria and animals mechanistically represent class I aldolases that form a Schiff-base intermediate with **4** [92] to promote *si*-face attack on the sugar aldehyde carbonyl group resulting in the formation of a (4*S*) configured stereocenter in the aldol product. Enzyme preparations from *Clostridium perfringens* and *E. coli* are commercially available, and the enzyme has broad pH optimum and useful stability at ambient temperature [93]. The functionally related 3-deoxy-*D*-manno-octulosonic acid aldolase (KdoA), which catalyzes *in vivo* the degradation of the 8-carbon sugar *D*-KDO (*ent*-**108**) into *D*-arabinose and **4**, has been less investigated [94]. The enzyme, in principle, is interesting for synthetic development because it creates a (4*R*) configured chiral center, which is opposite to that produced by NeuA. Also, the 2-keto-3-deoxy-6-phospho-*D*-gluconate aldolase (KDPG aldolase), which is produced by many bacteria for degradation of 6-phosphogluconate, has been probed for its synthetic utility [95–97].

Due to the importance of sialic acids in many biological recognition processes, NeuA has become popular for the chemoenzymatic synthesis of natural sialic acids and nonnatural derivatives thereof. The specificity of NeuA for **4** as nucleophilic substrate, apart from fluoropyruvate [98], seems to be absolute. On the other hand, the enzyme displays a fairly broad tolerance for analogs of the electrophilic substrate, such as a number of sugars and their derivatives larger or equal to pentoses (Table 13.3) [93,99,100]. Permissible variations tolerated by the aldolase include replacement of the natural *D*-manno configured substrate with derivatives containing modifications such as epimerization, substitution, or deletion at positions C-2, C-4, or C-6. Epimerization at C-2, however, is restricted to small polar substituents at strongly reduced reaction rates [101,102]. In search for viral neuraminidase inhibitors, many sialic acid analogs have been prepared containing modifications at C-5/C-9, such as *N*-acylated derivatives (e.g., **111**, **113**) [103–107], or those having modifications at C-9 (e.g., **112**, **114**) (Figure 13.19) [108–110]. 3-*N*-substituted mannosamine derivatives were not acceptable at all [111]. Examination of C-9 modified *N*-acetylneuraminic acid derivatives

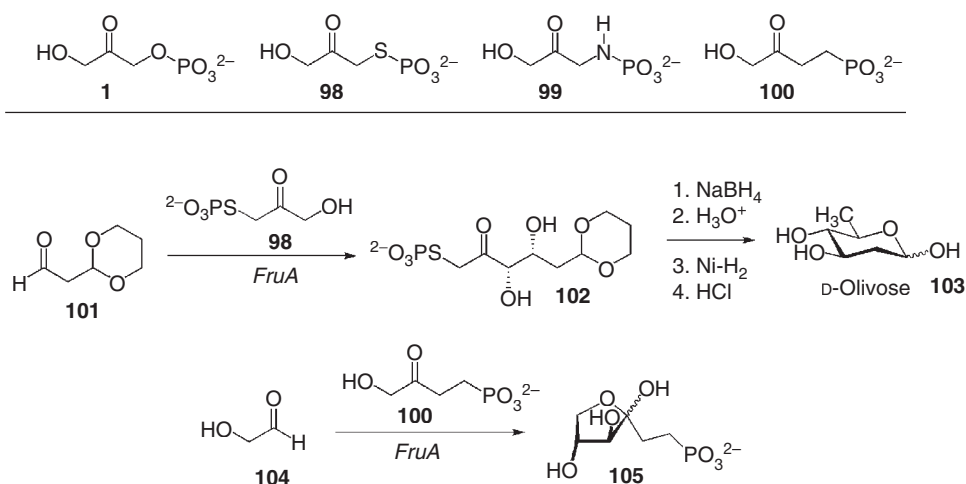


FIGURE 13.17 Substrate analogs of DHAP, and application in FruA-catalyzed aldolization reactions.

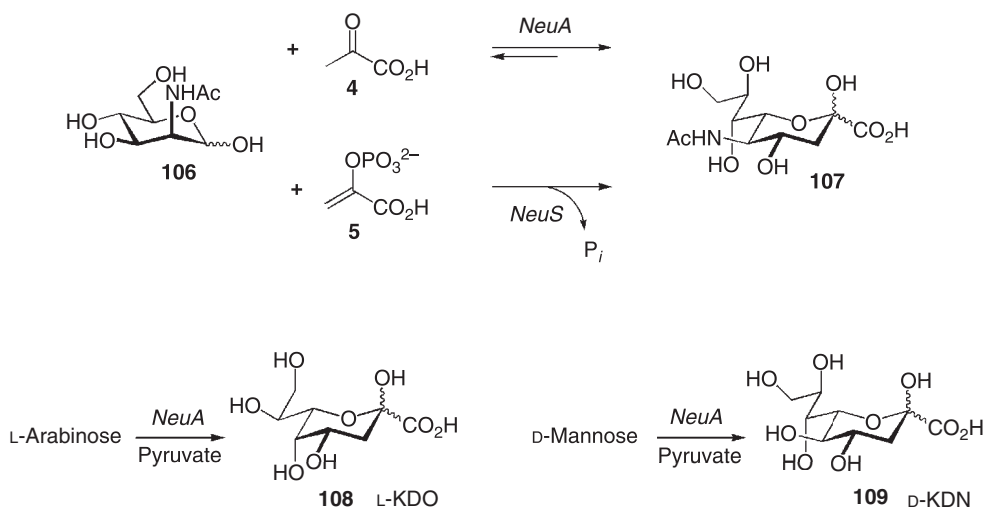


FIGURE 13.18 Synthesis of *N*-acetylneuraminic acid based on corresponding NeuA and NeuS catalysis, and preparation of other sialic acids on large scale by using NeuA.

TABLE 13.3
Substrate Tolerance of Neuraminic Acid Aldolase

R₁	R₂	R₃	R₄	R₅	Yield (%)	Relation Rate (%)
NHAc	H	OH	H	CH ₂ OH	85	100
NHAc	H	OH	H	CH ₂ OAc	84	20
NHAc	H	OH	H	CH ₂ OMe	59	—
NHAc	H	OH	H	CH ₂ N ₃	84	60
NHAc	H	OH	H	CH ₂ OP(O)Me ₂	42	—
NHAc	H	OH	H	CH ₂ O(L-lactoyl)	53	—
NHAc	H	OH	H	CH ₂ O(Gly- <i>N</i> -Boc)	47	—
NHAc	H	OH	H	CH ₂ F	22	60
NHAc	H	OMe	H	CH ₂ OH	70	—
NHAc	H	H	H	CH ₂ OH	70	—
NHC(O)CH ₂ OH	H	OH	H	CH ₂ OH	61	—
NHCbz	H	OH	H	CH ₂ OH	75	—
OH	H	OH	H	CH ₂ OH	84	91
OH	H	H	H	CH ₂ OH	67	35
OH	H	H	F	CH ₂ F	40	—
OH	H	OH	H	H	66	10
H	F	OH	H	CH ₂ OH	30	—
H	H	OH	H	CH ₂ OH	36	130
Ph	H	OH	H	CH ₂ OH	76	—

showed that they were accepted only at the expense of a reduced rate [110]. Similar to NeuA, KdoA showed high specificity for **4** as nucleophile, but displayed a broad substrate tolerance toward the electrophile [94]. As a notable distinction to NeuA, KdoA was also active on smaller acceptor substrates, such as glyceraldehyde.

Owing to the broad substrate spectrum of the NeuA and the fully reversible nature of the aldol addition reaction, the generation of a dynamic combinatorial library of sialic acid analogs from **4** and a few aldehyde precursors has been studied, which led to the amplification of products that were selectively binding to added wheat germ agglutinin [112].

In most cases investigated so far, a high level of asymmetric induction by NeuA for the (4*S*) configuration is retained. However, a number of carbohydrates were also found to be converted with random or even inverse stereoselectivity for the C-4 configuration (e.g., **115**, **116**) [100,113,114]. A critical and distinctive factor seems to be the recognition of the configuration by the enzymic catalyst at C-3 in the aldehydic substrate [99,100].

Apart from the synthesis of sialic acid related products, NeuA was also successfully employed in the synthesis of a possible precursor (**118**) for the synthesis of macrolide antibiotic amphotericin B (**119**) from 2-deoxy-2-*C*-hydroxymethyl-*D*-mannose (**117**) (Figure 13.20) [115,116].

Another elegant example for the use of NeuA in the synthesis of a nonsugar molecule is represented by the synthesis of 3-(hydroxymethyl)-6-*epi*-castanospermine (**121**) from *N*-(carbobenzyloxy)-*D*-mannosamine (**120**) (Figure 13.21) [117]. The family of polyhydroxy-indolizidine natural products, including castanospermine (**122**) and swainsonine (**123**), possess potent glycosidase inhibitor activity with possible applications in anticancer and anti-HIV therapy. It is remarkable that the sterically demanding *N*-substituent did not interfere with the enzyme catalytic function.

KDPG aldolase has been utilized for the highly stereoselective enzymatic synthesis of compound **126**, which represents the N-terminal amino acid part of the nucleoside antifungal nikkomycin K_x and K_z (Figure 13.22) [118]. In the one-pot two-step enzymatic synthesis, the stereoselective aldol reaction leading to **125** is followed by a reductive amination, catalyzed by phenylalanine dehydrogenase, with high overall yield (75.9%) and enantiomeric excess (>99.7%).

Zanamivir (**129**) was introduced by Glaxo, in 1999, for the treatment of influenza infection, which represented the first medicinally used sialic acid derivative with antiviral activity. This required the cost-effective synthesis of the rare and expensive *N*-acetylneuraminic acid (**107**), for which NeuA from *E. coli* was the first aldolase to find application in an industrial process at a multiton scale [119–121].

In a first approach, the expensive *N*-acetylmannosamine (**106**) was produced by combining an enzymatic *in situ* isomerization of inexpensive *N*-acetylglucosamine (**128**) catalyzed by *N*-acetylglucosamine 2-epimerase to the NeuA reaction in an enzyme membrane reactor (Figure 13.23) [122–124]. Unfortunately, both the steps in the sequence are fully reversible and are limited by unfavorable equilibrium constants (e.g., equilibrium constant of 12.7 M^{-1} in favor of the retroaldol reaction [19]), which mandates excess of **4** (usually sevenfold to tenfold) to achieve a preparative useful conversion. Product isolation, however, is complicated by the excess of **4**, and usually requires purification by ion exchange chromatography.

As an other example of this continuous process design, *D*-KDN (**109**) has been produced on a 100-g scale from *D*-mannose and **4** using a pilot-scale enzyme membrane reactor with a space-time yield of $375\text{ g L}^{-1}\text{ d}^{-1}$ and an overall crystallized yield of 75% [125]. In analogy to the *D*-KDN process, nonnatural *L*-KDO (**108**) has been prepared using *L*-arabinose in place of *D*-mannose (Figure 13.18) [99].

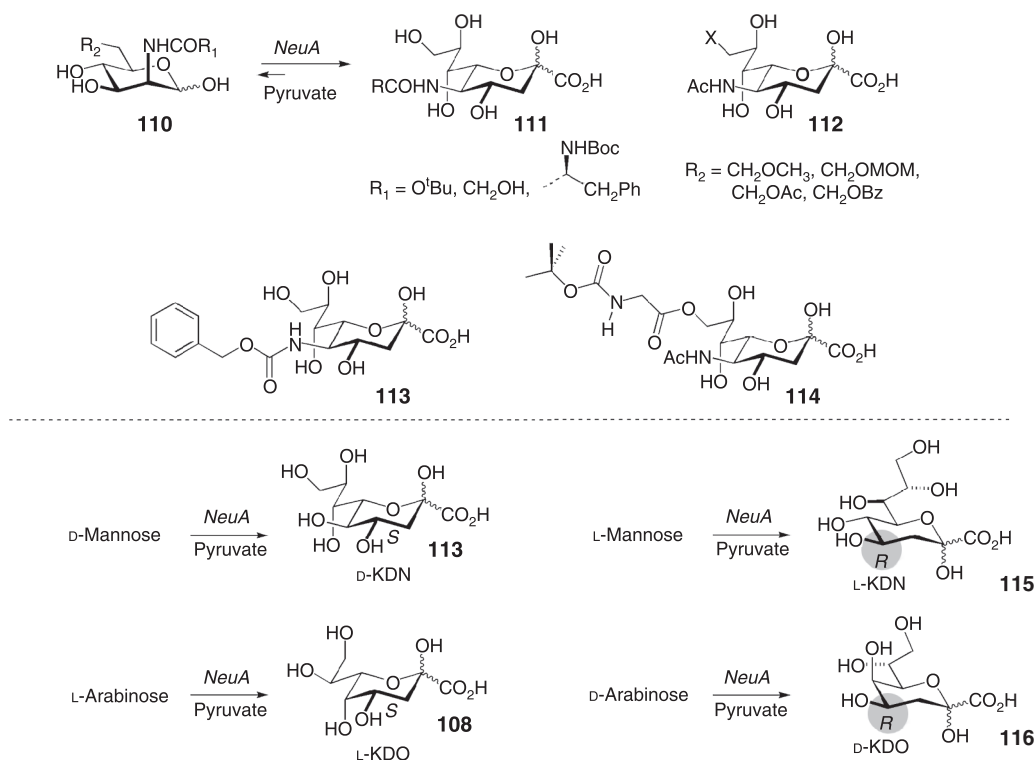


FIGURE 13.19 Examples of neuraminic acid derivatives accessible by NeuA catalysis.

In an alternative approach, serviceably high concentration of **106** may be achieved if the latter were to be produced separately by chemical base catalysis [119,121]. Also, it was found that product recovery can be facilitated by removing residual **4** through formation of a separable bisulfite adduct [121] or by decomposing **4** into volatile compounds using yeast pyruvate decarboxylase [113,119].

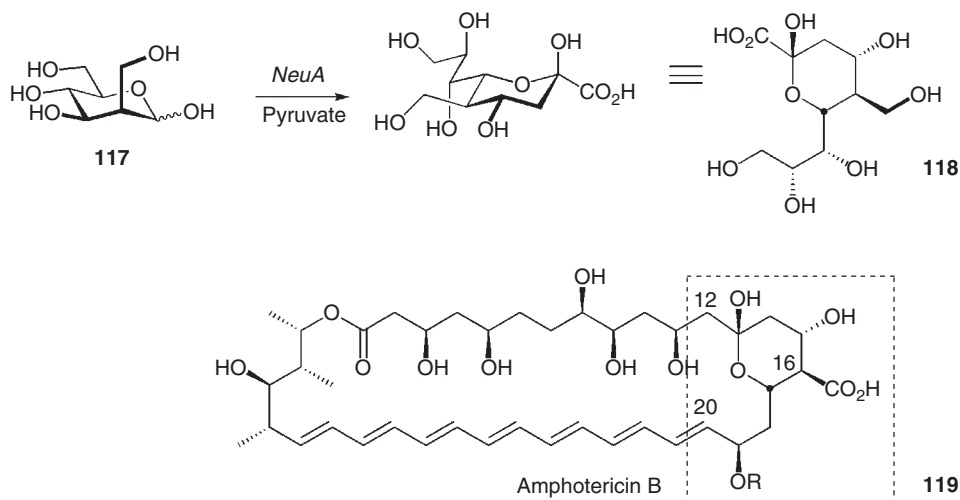


FIGURE 13.20 NeuA-catalyzed synthesis of a possible synthetic precursor for the macrolide antibiotic amphotericin B.

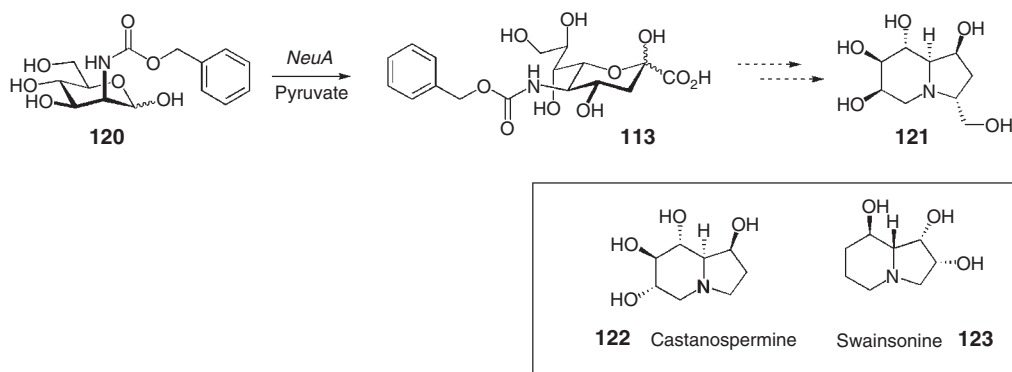


FIGURE 13.21 NeuA-based chemoenzymatic synthesis of the polyhydroxylated indolizidine analog 3-(hydroxymethyl)-6-epicastanospermine.

The need of excess **4** might be circumvented altogether when the NeuA-catalyzed synthesis of **107** is coupled to a thermodynamically favored process, such as an irreversible sialyltransfer reaction. This option has been realized with a multienzyme system for a sialyltransferase-catalyzed synthesis of sialyloligosaccharides [126–128], where the driving force is provided by consumption of **107** in the CTP-dependent activation to give the glycosyl donor CMP-Neu5NAc, and by irreversible *in situ* regeneration of the nucleotide triphosphate (Figure 13.24). This approach was also recently employed by the one-pot chemoenzymatic synthesis of a variety of CMP-sialic acid derivatives **132** from various analogs of **106** (e.g., **130**; Figure 13.24) [129]. This and earlier approaches demonstrate the substrate flexibility of not only the neuraminic acid aldolase, but also the CMP-sialic acid synthases that were employed in the synthesis reactions [130].

13.5 2-DEOXYRIBOSE 5-PHOSPHATE ALDOLASE

The family of aldehyde-dependent aldolases comprises only one member known so far, the 2-deoxyribose 5-phosphate aldolase (DerA or RibA, EC 4.1.2.4). *In vivo* DerA catalyses the

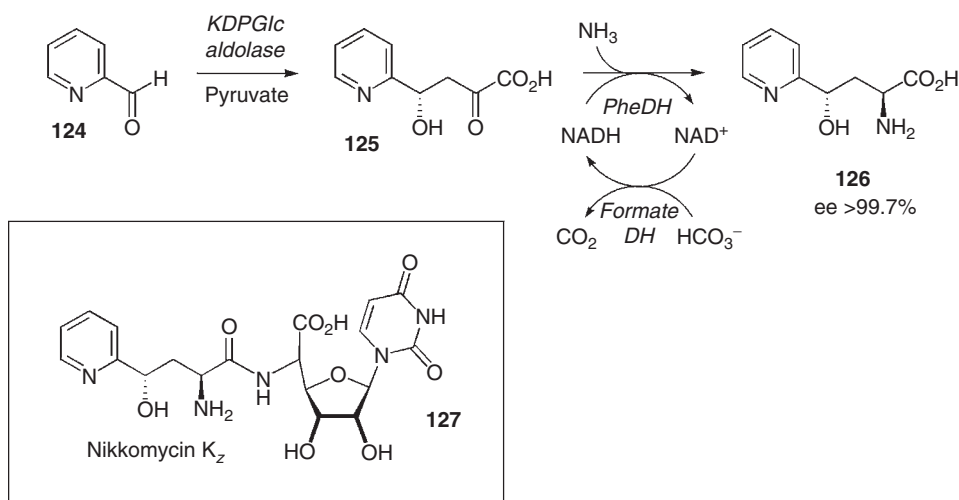


FIGURE 13.22 Chemoenzymatic synthesis of the arylated amino acid part of the nucleoside antifungal nikkomycins K_x and K_z using KDPG aldolase.

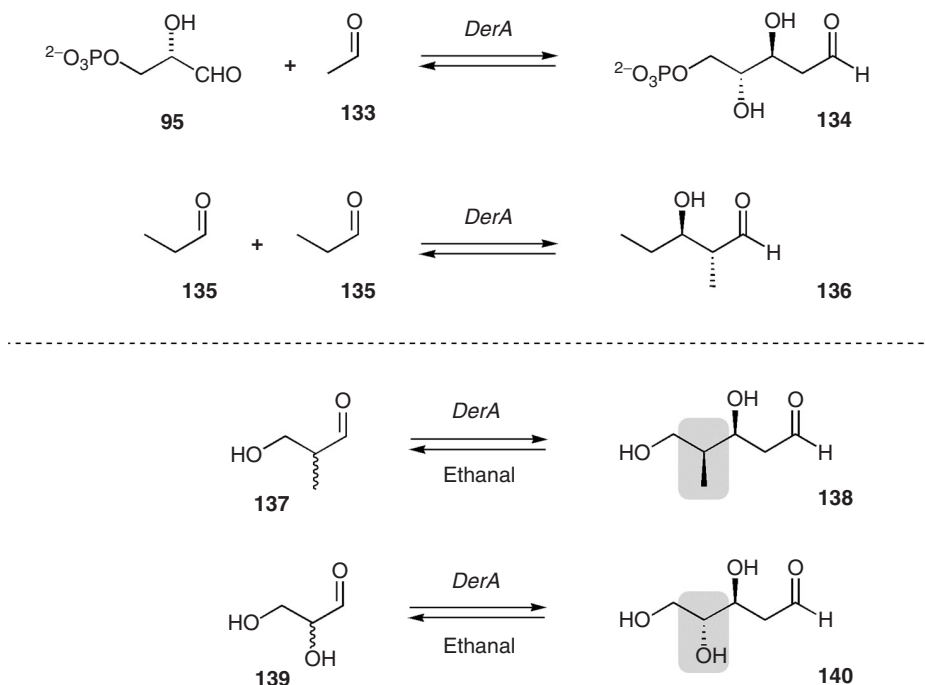


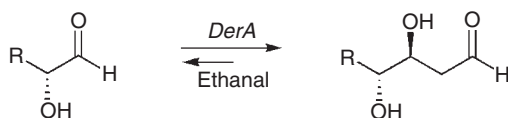
FIGURE 13.25 DerA-catalyzed stereoselective aldolization reactions.

lysine residue during the catalytic cycle [131]. DerA from *E. coli* can be easily overexpressed by homologous overexpression in *E. coli*, and the crude cell-free extract often qualifies for preparative applications without further purification [132–134]. The enzyme is rather stable under reaction conditions and at room temperature.

Like other aldolases, DerA is rather specific for its physiological donor substrate, but instead of **133** also the structural closely related propanal (**135**), acetone, or fluoroacetone are acceptable [132,133]. The substrate tolerance for acceptor aldehydes is rather relaxed, and many aliphatic and variously substituted aldehydes can be used as a replacement for the natural substrate **95**, albeit at the expense of strongly reduced catalytic rates (Table 13.4) [132,133]. The enzyme shows high stereoselectivity for the newly generated chiral center at C3; remarkably, **135** as a donor substrate yielded only a single diastereomer of absolute (2*R*,3*S*) configuration [133] (Figure 13.25). This is indicative not only of the high level of asymmetric induction at the acceptor carbonyl, but also of the stereospecific deprotonation of the aldol donor substrate. Interestingly, acceptor aldehydes possessing a small hydrophobic group at C2 (e.g., **137**) with L-configuration were strongly preferred by DerA to yield *syn* adducts (**138**), whereas the D-isomers were the preferred substrate in case of polar groups, such as OH (**139**) or *N*₃ substituents [135,136].

Corresponding to its natural function, DerA has been used for the synthesis of ¹³C-labeled 2-deoxy-D-ribose from labeled **133** and **95** [137], and in a commercial multienzyme process for the synthesis of purine and pyrimidine containing deoxyribonucleosides (e.g., **142**; Figure 13.26) [138,139]. Synthetic applications of DerA also include the preparation of various sugar derivatives, such as 2-deoxy, thio, and aza sugars [133]. Specifically, DerA was used for the stereoselective synthesis of 2-deoxy-L-fucose, a constituent of several antibiotics, starting from (2*R*,3*S*)-dihydroxybutanal and **133** [134]. Recently, DerA has been used in the stereocontrolled addition of **133** to *rac*-lactaldehyde and (*S*)-2-methyl-3-hydroxypropanal (**143**) to furnish chiral building blocks (e.g., **144–146**) that are useful for the synthesis of anticancer agents epothilone A (**147**) and C (Figure 13.27) [135].

TABLE 13.4
Substrate Spectrum Accepted by *E. coli* 2-Deoxy-D-Ribose-5-Phosphate Aldolase



R	Yield (%)	Relation Rate (%)
CH ₂ OPO ₃ ²⁻	78	100
H	20	—
CH ₂ OH	65	0.4
CH ₃	32	0.4
CH ₂ F	33	0.4
CH ₂ Cl	37	0.3
CH ₂ Br	30	—
CH ₂ SH	33	—
CH ₂ N ₃	76	0.3
C ₂ H ₅	18	0.3
CH=CH ₂	12	—
CHOH-CH ₂ OH	62	0.3
CHN ₃ -CH ₂ OH	46	—
CHOH-CH ₃	51	—
CHOH-CH ₂ -C ₆ H ₅	46	—
CH ₂ SCH ₂ -CHOH-CH ₂ OH	27	—

An interesting transformation is the sequential asymmetric addition of two equivalents of donor **133** to a starting acceptor unit (Figure 13.28). In such a cascade, which is observed with simple α -substituted ethanal derivatives that contain a functionality unable to induce

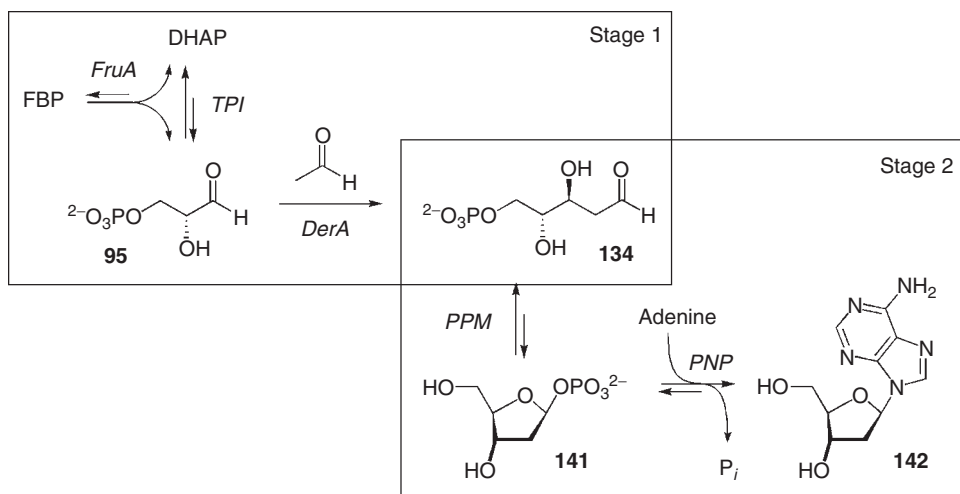


FIGURE 13.26 Two-stage aldolase-based process used for the preparative scale synthesis of deoxynucleosides.

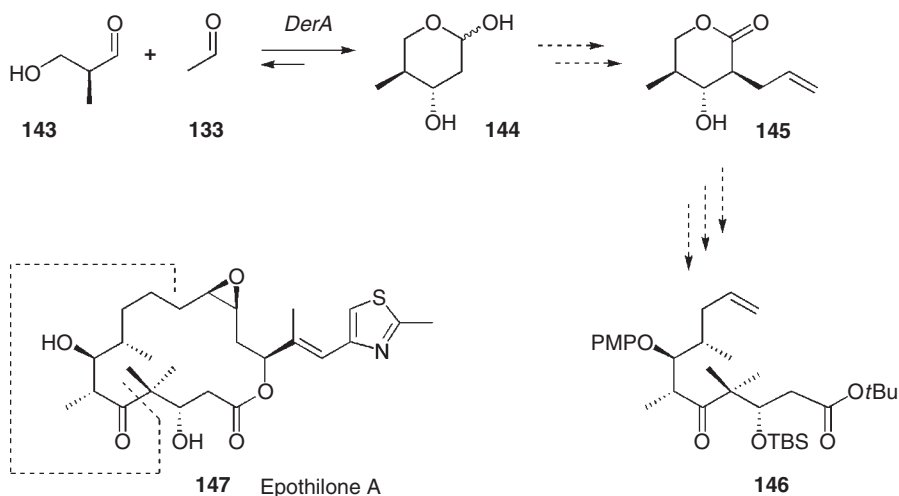


FIGURE 13.27 Chemoenzymatic synthesis of a chiral epothilone A building block using DerA-catalyzed aldolization reaction.

cyclization of the initial product (e.g., **149**) to a hemiacetal, the first aldol product acts as an acceptor substrate for a second aldol addition. Only at this stage (e.g., **150**) a cyclization can happen, which yields stable pyranoses and thereby prevents further aldol steps to take place [140]. A particular good acceptor substrate is chloroethanal (**148**), which yields

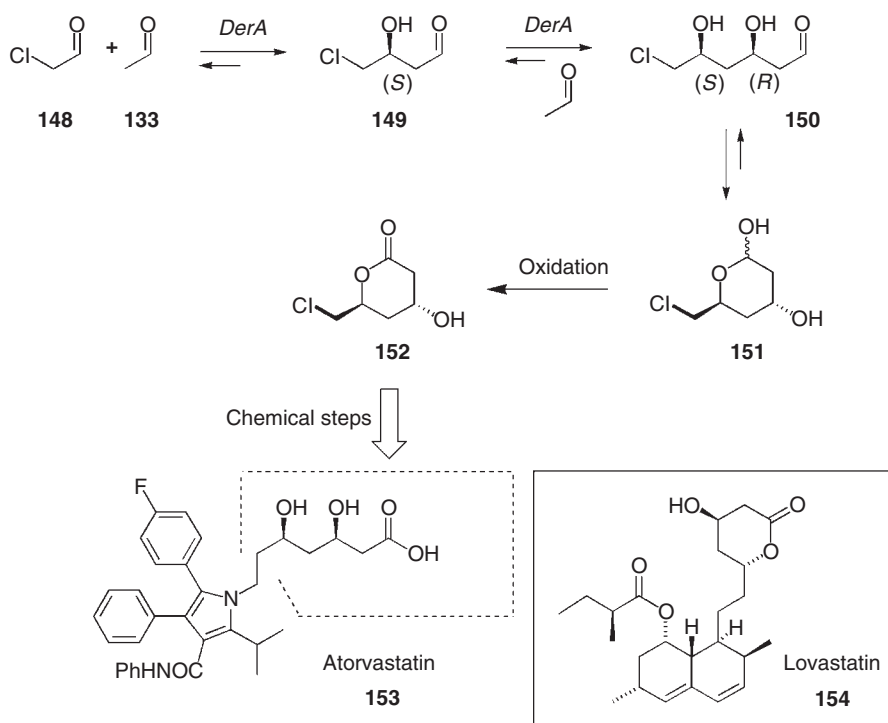


FIGURE 13.28 DerA-catalyzed sequential aldol reaction yielding a key chiral building block for the cholesterol lowering vastatin drugs.

(3*R*,5*S*)-6-chloro-2,4,6-trideoxyhexapyranoside (**151**), a useful building block with two chiral centers for 1,3-polyol containing natural products. Recently, this reaction has been developed into an industrial process for the large-scale synthesis of the chiral side chain of the statins [141–143], which are marketed as important HMG-CoA reductase inhibitors. Although several elegant chemoenzymatic synthetic routes have been developed for such skipped polyols [144], the aldol route to this polyfunctional chiral building block (**152**) is economically most attractive because the asymmetric centers are created without cofactor demand directly from cheap bulk chemicals.

13.6 GLYCINE-DEPENDENT ALDOLASES

The metabolism of β -hydroxy- α -amino acids involves pyridoxal phosphate-dependent enzymes, classified as serine hydroxymethyltransferase (SHMT) and threonine aldolase (ThrA). Both enzymes catalyze reversible aldol-type cleavage reactions yielding glycine (**156**) and an aldehyde (Figure 13.29). Whereas SHMT *in vivo* has a biosynthetic function, ThrA catalyzes the degradation of threonine (**158**, **159**) to **156** and **133**; both L- and D-specific ThrA enzymes are known [9,145]. Typically, ThrA enzymes show complete enantioselectivity for their natural α -D- or α -L-amino configuration but, with few exceptions, have only low specificity for the relative *threo/erythro*-configuration (e.g., **158/159**) [146]. Likewise, SHMT is highly selective for L-configuration, but has poor *threo/erythro*-selectivity [147].

For biocatalytic applications, the known SHMT, D- and L-ThrA show broad substrate tolerance for various acceptor aldehydes (Table 13.5), including aromatic aldehydes [146–148]; however, α,β -unsaturated aldehydes are not accepted [149].

β -Hydroxyamino acids constitute an important class of compounds, representing natural products of their own right, like L-threonine (**158**, **159**) or L-serine (**157**), or components of more complex structures, such as the β -hydroxytyrosine moiety present in vancomycin [150]. The synthesis of **157** has been developed on multimolar scale using SHMT from *Klebsiella aerogenes* or *E. coli* to furnish the product at high final concentration of >450 g/L [151–154]. SHMT has also been employed for the synthesis of L-*erythro*-2-amino-3-hydroxy-1,6-hexanedicarboxylic acid **160** [155], a potential precursor for carbocyclic β -lactams and nucleotides (Figure 13.30). The *erythro*-selective L-ThrA from the yeast *Candida humicola* has been used for the synthesis of (*S,S,R*)- and (*S,S,S*)-3,4-dihydroxyprolines (**167**, **168**) [156], and for the preparation of a chiral building block (**162**) toward the synthesis of the immunosuppressive lipid mycostericin D (**163**) [157].

Due to the fully reversible equilibrium nature of the aldol addition process, enzymes with low diastereoselectivity will typically lead to a thermodynamically controlled mixture of

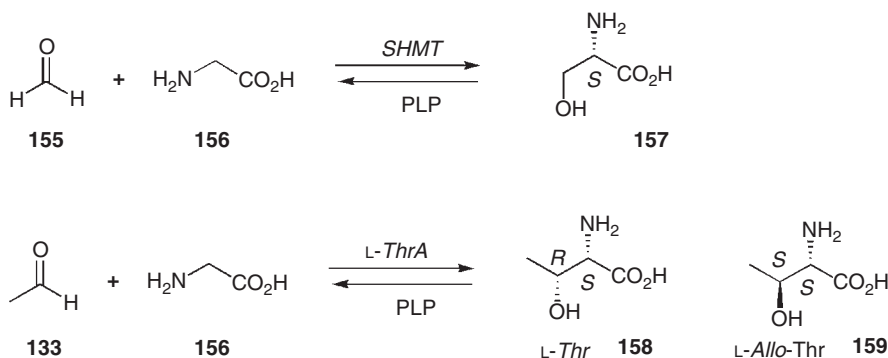


FIGURE 13.29 Aldol reactions catalyzed *in vivo* by SHMT and threonine aldolases.

TABLE 13.5
Substrate Tolerance of SHMT and Threonine Aldolases

R	SHMT Selectivity (threo/erythro)	Yield (%)	L-ThrA Selectivity (threo/erythro)	Yield (%)	D-ThrA Selectivity (threo/erythro)	Yield (%)
H	—	94				
CH ₃	2:98	—	9 : 91	40	53 : 47	60
C ₃ H ₉			24 : 76	21	67 : 33	37
C ₅ H ₁₁	60:40	25	37 : 63	16	61 : 39	31
CH(CH ₃) ₂			46 : 53	15	84 : 16	26
CH ₂ OBn			2 : 98	88		
CH ₂ CH ₂ OBn			47 : 53	53		
(CH ₂) ₂ C ₆ H ₅			28 : 72	10	87 : 13	16
C ₆ H ₅	60:40	22	60 : 40	9	74 : 26	11
<i>p</i> -C ₆ H ₄ NO ₂			47 : 53	53	55 : 45	88
<i>o</i> -C ₆ H ₄ NO ₂			58 : 42	93	72 : 28	89
<i>m</i> -C ₆ H ₄ OH			73 : 27	43	74 : 26	54
<i>p</i> -C ₆ H ₄ CH ₃			55 : 45	17	43 : 57	25
2-Imidazolyl	33:67	10	66 : 34	40	61 : 39	60
2-Turanyl	50:50	20				
2-Thienyl	56:44	11				

threo/erythro-isomers that are difficult to separate, e.g., by fractional crystallization or ion-exchange chromatography. A further problem is that the overall equilibrium constant does not favor synthesis [19], which requires the reactions to be driven by an excess of **156** (which is difficult to separate from the product) or the aldehyde (which at high concentration may engage in condensation reactions or inactivate the enzyme). Therefore, members of this class of aldolases have been more successfully applied in the kinetic resolution of racemic mixtures of β -hydroxy amino acids produced by chemical synthesis.

In this respect, SHMT has been used to resolve racemic *erythro* β -hydroxy amino acids to produce pure D-*erythro* isomers [158,159]. An L-ThrA from *Streptomyces amakusaensis* was found to be particularly useful for the resolution of racemic *threo*-aryl serines (**169**), yielding enantiomerically pure D-amino acids (e.g., **170**; Figure 13.31) [155,160]. As a complementary example, the recombinant low-specificity D-ThrA of *Arthrobacter* sp. DK-38 was used for the preparation of L-*threo*- β -(4-methylthiophenyl)serine (**173**) [161] and L-*threo*- β -(3,4-methylenedioxyphenyl)serine (**176**) from DL-*threo*-isomeric mixtures [162]. The former represents an intermediate in the synthesis of the antibiotics florfenicol and thiamphenicol, while the latter serves as an intermediate for the production of a Parkinson drug.

13.7 TRANSKETOLASE

Transketolase (EC2.2.1.1) is an enzyme synthetically relevant to, but mechanistically distinct from, the aldolases for asymmetric C–C bond forming reactions. *In vivo*, the enzyme catalyzes

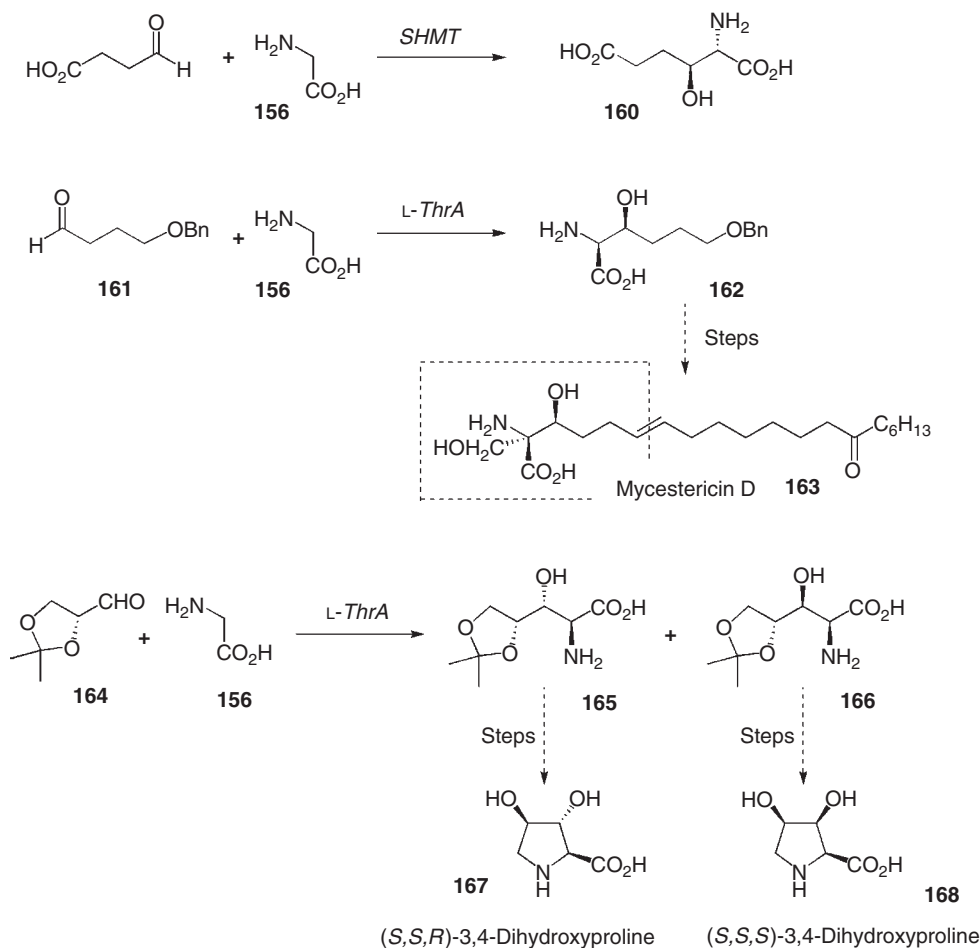


FIGURE 13.30 Synthetic application of *ThrA* for stereoselective synthesis of a potential chiral building block for the immunosuppressive lipid mycestericin D, and of dihydroxyprolines from glyceraldehyde.

the reversible transfer of a 2-carbon ketol unit between phosphorylated intermediates of the oxidative pentose phosphate pathway (Figure 13.32). Its mechanism resembles a classical benzoin addition, and the enzyme requires thiamine diphosphate and divalent Mg as cofactors [163]. For preparative reactions, the natural ketol donor D-xylulose 5-phosphate (**178**) [164] can be replaced by hydroxypyruvate (**182**) [165], which makes the enzyme more generally useful and renders synthetic reactions irreversible due to the spontaneous decarboxylation of the reactive intermediate (Figure 13.33). This feature compensates for the significant rate reduction with **182** as a nonphysiological substrate; also **182** causes no inhibition of the enzyme even at high concentrations [166].

Transketolase from different sources has been shown to possess a broad acceptor substrate spectrum, yielding products with complete (*S*)-stereospecificity for the newly formed chiral center [165]. Although generic aldehydes are converted with full stereocontrol and even α,β -unsaturated aldehydes are accepted to some degree, hydroxylated electrophiles are usually converted with higher rates [167]. In the latter case, transketolase discriminates 2-hydroxyaldehydes (**181**) with complete kinetic preference for the (*R*)-configuration to yield enantiopure diols of (*3S,4R*)-configuration (**183**; Figure 13.33) [168,169].

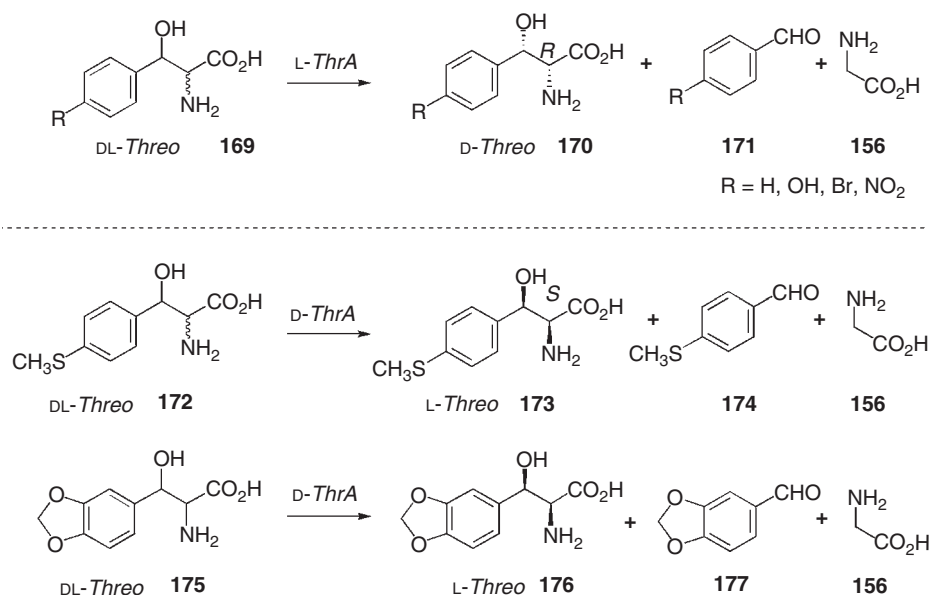


FIGURE 13.31 ThrA-catalyzed resolution of diastereomeric mixtures by retroaldolization under kinetic control yielding enantiomerically pure arylserines.

Recently, the transketolase of *E. coli* has been developed for large-scale enzyme production [170–172]. The enzyme is quite tolerant to organic cosolvents, and its immobilization stabilizes significantly against inactivation by aldehyde substrates [173]. Continuous pilot reactions have been performed in a membrane reactor [172], and *in situ* product removal through borate complexation has been probed [174].

Synthetic applications of transketolase include the preparation of valuable ketose sugars, such as fructose analogs [165]. In addition, transketolase has been utilized for the key steps in the chemoenzymatic synthesis of (+)-exo-brevicomin (**72**; Figure 13.34) [60], azasugars such as 1,4-dideoxy-1,4-imino-D-arabinol [53] and fagomine [175], or *N*-hydroxypyrrolidine (**188**) [176] from 3-azido, 3-cyano, and 3-*O*-benzyl (**186**) analogs and derivatives of glyceraldehyde, respectively. All syntheses take advantage of the intrinsic kinetic resolution

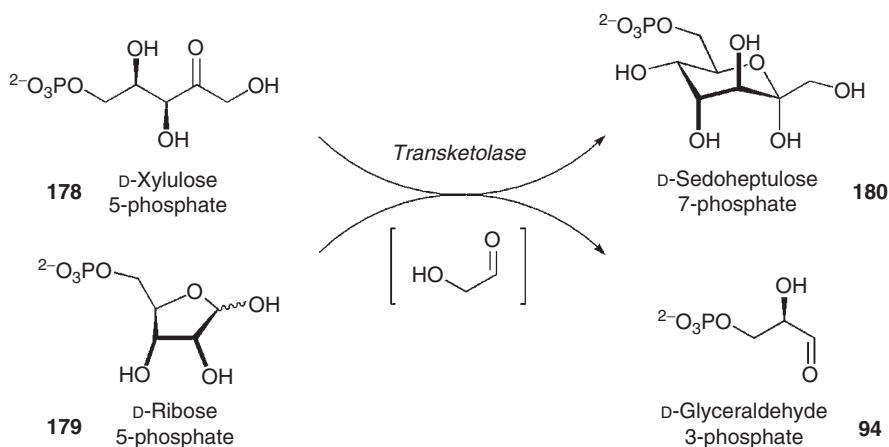


FIGURE 13.32 *In vivo* reaction catalyzed by transketolase.

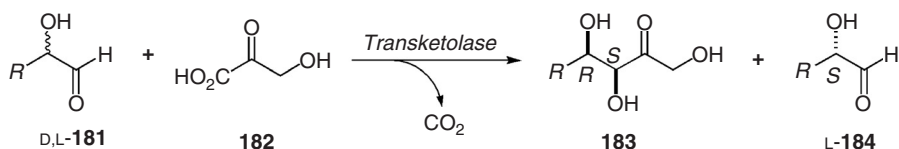


FIGURE 13.33 Kinetic resolution catalyzed by transketolase, and nonequilibrium C–C bond formation by decomposition of hydroxypyruvate.

of 2-hydroxyaldehydes by the enzyme and the thermodynamic driving force from the decarboxylation of **182**. Even the option to prepare (*S*)-configured 2-hydroxyaldehydes (**184**) by consumption of the (*R*)-antipodes from the racemate in a transketolase reaction (Figure 13.33) has been verified [169]. More recently, transketolase has been utilized to prepare a fluorogenic substrate (**190**), useful for the high-throughput screening of stereoselective transaldolases (Figure 13.34) [177].

13.8 PERSPECTIVES

Aldol addition reactions are one among the most fundamental methodologies available to the synthetic chemist for the construction of new C–C bonds. Therefore, control of the stereochemical course of the aldol reaction for the preparation of enantiomerically and diastereomerically pure products has attracted considerable interest, and asymmetric aldol reactions are clearly some of the best-developed organic transformations today [5,178,179]. This chapter has attempted to demonstrate the remarkable progress of asymmetric enzymatic aldol reactions by highlighting the state of development of readily available enzymes, the most important synthetic examples, and the most efficient reaction techniques. This clearly proves that biocatalytic C–C bond formation is eminently useful, and highly predictable, for the asymmetric synthesis of complex multifunctional molecules. It is also evident that the

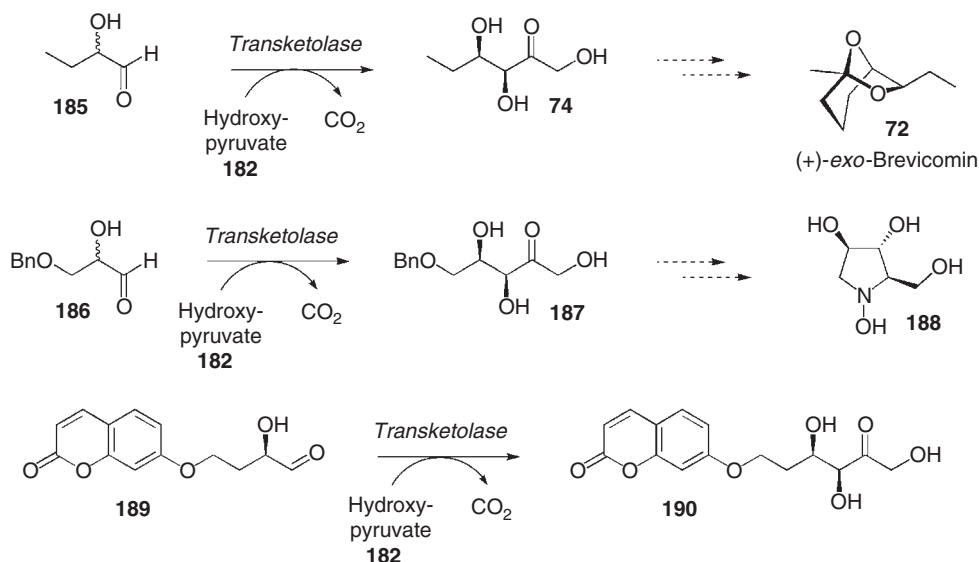


FIGURE 13.34 Chemoenzymatic synthesis of (+)-*exo*-brevicomin, an *N*-hydroxypyrolidine, and a fluorescence screening substrate based on stereospecific transketolase catalysis.

technology is well accepted in the chemical community and, with several examples for large-scale industrial processes now in operation, that the field is maturing rapidly.

Enzyme-catalyzed asymmetric aldol reactions are not only highly efficient, simple to operate even at large scale, but can also be extremely cost-effective and environmental friendly [143]. Clearly, in comparison with current chemical methods for asymmetric aldol reactions, the ecological advantage of biocatalytic procedures stems from the fact that they do not suffer from a need for harsh reaction conditions, corrosive reagents, costly protecting groups, high catalyst loading, consumption of chiral auxiliaries, or heavy metal leakage to products. Despite a broad scope of synthetic opportunities, however, it becomes obvious that enzymatic aldol reactions still suffer from restrictions that mostly derive from the limited flexibility of applicable donor components, or from the costs inherent in their preparation. To remain competitive with modern chemical developments such as proline-based organocatalysis [180], current focus is therefore at lifting the restraints and tailoring aldolases for specific needs by different approaches, such as by rational protein engineering based on the host of available protein crystal structures [6] or by directed evolution through random mutagenesis [181,182]. Catalytic aldolase antibodies still suffer from inconveniently low catalytic rates [183,184], but they may guide in the construction of aldolases that act on more generic, less functionalized substrates. Prospecting for novel activities through whole-genome sequencing efforts, mass screening of previously untapped natural [185,186], or panning of man-made biodiversity may furnish attractive biocatalysts for new applications of C–C bond formation in biotechnology [187].

REFERENCES

1. Collins, A.N., Sheldrake, G.N., and Crosby, J., *Chirality in Industry*, Wiley, Chichester, vol. 1, 1992, vol. 2, 1997.
2. Schmid, A., Dordick, J.S., Hauer, B., et al., Industrial biocatalysis today and tomorrow, *Nature*, 409, 258–268, 2001.
3. Schoemaker, H.E., Mink, D., and Wubbolts, M.G., Dispelling the myths—biocatalysis in industrial synthesis, *Science*, 299, 1694–1697, 2003.
4. Liese, A., Seelbach, K., and Wandrey, C., *Industrial Biotransformations*, Wiley-VCH, Weinheim, 2000.
5. Mahrwald, R., *Modern Aldol Reactions*, Wiley-VCH, Weinheim, Germany, 2004.
6. Fessner, W.-D., Enzyme-catalyzed aldol additions, in *Modern Aldol Reactions*, vol. 1, Mahrwald, R., Ed., Wiley-VCH, Weinheim, Germany, 2004, pp. 201–272.
7. Griengl, H., Schwab, H., and Fechter, M., The synthesis of chiral cyanohydrins by oxynitrilases, *Trends Biotechnol.*, 18, 252–256, 2000.
8. Pohl, M., Lingen, B., and Müller, M., Thiamin-diphosphate-dependent enzymes: new aspects of asymmetric C–C bond formation, *Chem. Eur. J.*, 8, 5288–5295, 2002.
9. Fessner, W.-D. and Walter, C., Enzymatic C–C bond formation in asymmetric synthesis, *Top. Curr. Chem.*, 184, 97–194, 1996.
10. Silvestri, M.G., Desantis, G., Mitchell, M., et al., Asymmetric aldol reactions using aldolases, *Top. Stereochem.*, 23, 267–342, 2003.
11. Hildebrandt, G. and Klavehn, W., L-1-Phenyl-2-methylamino-1-propanol, US 1956950: 1934 (*Chem. Abstr.* 1934, 28, 40723).
12. Waldmann, H. and Sebastian, D., Enzymatic protecting group techniques, *Chem. Rev.*, 94, 911–937, 1994.
13. Horecker, B.L., Tsolas, O., and Lai, C.Y., Aldolases, in *The Enzymes*, vol. VII, Boyer, P.D., Ed., Academic Press, New York, 1972, pp. 213–258.
14. Fessner, W.-D., Schneider, A., Held, H., et al., The mechanism of class II, metal-dependent aldolases, *Angew. Chem. Int. Ed. Engl.*, 35, 2219–2221, 1996.
15. Bednarski, M.D., Simon, E.S., Bischofberger, N., et al., Rabbit muscle aldolase as a catalyst in organic synthesis, *J. Am. Chem. Soc.*, 111, 627–635, 1989.

16. von der Osten, C.H., Sinskey, A.J., Barbas, C.F., et al., Use of a recombinant bacterial fructose-1,6-diphosphate aldolase in aldol reactions: preparative syntheses of 1-deoxynojirimycin, 1-deoxymannojirimycin, 1,4-dideoxy-1,4-imino-D-arabinitol, and fagomine, *J. Am. Chem. Soc.*, 111, 3924–3927, 1989.
17. Fessner, W.-D., Sinerius, G., Schneider, A., et al., Diastereoselective enzymatic aldol additions: L-rhamnulose and L-fuculose 1-phosphate aldolases from *E. coli.*, *Angew. Chem. Int. Ed. Engl.*, 30, 555–558, 1991.
18. Zannetti, M.T., Walter, C., Knorst, M., et al., Fructose 1,6-bisphosphate aldolase from *Staphylococcus carnosus*: overexpression, structure prediction, stereoselectivity, and application in the synthesis of bicyclic sugars, *Chem. Eur. J.*, 5, 1882–1890, 1999.
19. Goldberg, R.N. and Tewari, Y.B., Thermodynamics of enzyme-catalyzed reactions: Part 4. Lyases, *J. Phys. Chem. Ref. Data*, 24, 1669–1698, 1995.
20. Brinkkotter, A., Shakeri, G.A., and Lengeler, J.W., Two class II D-tagatose-bisphosphate aldolases from enteric bacteria, *Arch. Microbiol.*, 177, 410–419, 2002.
21. Ghalambor, M.A. and Heath, E.C., L-Fuculose 1-phosphate aldolase, *Meth. Enzymol.*, 9, 538–542, 1966.
22. Takagi, Y., L-Rhamnulose 1-phosphate aldolase, *Meth. Enzymol.*, 9, 542–545, 1966.
23. Chiu, T.H., Evans, K.L., and Feingold, D.S., L-Rhamnulose-1-phosphate aldolase, *Meth. Enzymol.*, 42, 264–269, 1975.
24. Durany, O., de Mas, C., and Lopez-Santin, J., Fed-batch production of recombinant fuculose-1-phosphate aldolase in *E. coli.*, *Process Biochem.*, 40, 707–716, 2005.
25. Vidal, L., Durany, O., Suau, T., et al., High-level production of recombinant his-tagged rhamnulose 1-phosphate aldolase in *Escherichia coli.*, *J. Chem. Technol. Biotechnol.*, 78, 1171–1179, 2003.
26. Toone, E.J., Simon, E.S., Bednarski, M.D., et al., Enzyme-catalyzed synthesis of carbohydrates, *Tetrahedron*, 45, 5365–5422, 1989.
27. Bissett, D.L. and Anderson, R.L., Lactose and D-galactose metabolism in *Staphylococcus aureus*. IV. Isolation and properties of a class I D-ketohexose-1,6-diphosphate aldolase that catalyzes the cleavage of D-tagatose 1,6-diphosphate, *J. Biol. Chem.*, 255, 8750–8755, 1980.
28. Fessner, W.-D. and Eyrisch, O., One-pot synthesis of tagatose 1,6-bisphosphate via diastereoselective enzymic aldol addition, *Angew. Chem. Int. Ed. Engl.*, 31, 56–58, 1992.
29. Eyrisch, O., Sinerius, G., and Fessner, W.-D., Facile enzymic *de novo* synthesis and NMR spectroscopic characterization of D-tagatose 1,6-bisphosphate, *Carbohydr. Res.*, 238, 287–306, 1993.
30. Williams, G.J., Domann, S., Nelson, A., et al., Modifying the stereochemistry of an enzyme-catalyzed reaction by directed evolution, *Proc. Natl. Acad. Sci. USA*, 100, 3143–3148, 2003.
31. Fessner, W.-D., Badia, J., Eyrisch, O., et al., Enzymic syntheses of rare ketose 1-phosphates, *Tetrahedron Lett.*, 33, 5231–5234, 1992.
32. Fessner, W.-D., Schneider, A., Eyrisch, O., et al., 6-Deoxy-L-lyxo- and 6-deoxy-L-arabino-hexulose 1-phosphates. Enzymic syntheses by antagonistic metabolic pathways, *Tetrahedron Asymmetry*, 4, 1183–1192, 1993.
33. Lees, W.J. and Whitesides, G.M., Diastereoselectivity (enantioselectivity) of aldol condensations catalyzed by rabbit muscle aldolase at C-2 of RCHOHCHO if R has an appropriately placed negatively charged group, *J. Org. Chem.*, 58, 1887–1894, 1993.
34. Durrwachter, J.R., Wong, C.-H., Fructose 1,6-diphosphate aldolase-catalyzed stereoselective synthesis of C-alkyl and N-containing sugars: thermodynamically controlled C–C bond formations, *J. Org. Chem.*, 53, 4175–4181, 1988.
35. Kim, M.-J., Lim, I.T., Synthesis of unsaturated C8–C9 sugars by enzymatic chain elongation, *Synlett*, 138–140, 1996.
36. Serianni, A.S., Cadman, E., Pierce, J., et al., Enzymic synthesis of carbon-13-enriched aldoses, ketoses, and their phosphate esters, *Meth. Enzymol.*, 89, 83–92, 1982.
37. Wong, C.-H. and Whitesides, G.M., Synthesis of sugars by aldolase-catalyzed condensation reactions, *J. Org. Chem.*, 48, 3199–3205, 1983.
38. Fessner, W.-D. and Sinerius, G., Synthesis of dihydroxyacetone phosphate (and isosteric analogs) by enzymatic oxidation: sugars from glycerol, *Angew. Chem. Int. Ed. Engl.*, 33, 209–212, 1994.

39. Franke, D., Machajewski, T., Hsu, C.-C., et al., One-pot synthesis of L-fructose using coupled multienzyme systems based on rhamnulose-1-phosphate aldolase, *J. Org. Chem.*, 68, 6828–6831, 2003.
40. Fessner, W.-D. and Walter, C., “Artificial metabolisms” for the asymmetric one-pot synthesis of branched-chain saccharides, *Angew. Chem. Int. Ed. Engl.*, 31, 614–616, 1992.
41. Liu, K.K.C. and Wong, C.-H., A new strategy for the synthesis of nucleoside analogues based on enzyme-catalyzed aldol reactions, *J. Org. Chem.*, 57, 4789–4791, 1992.
42. Wong, C.-H., Moris-Varas, F., Hung, S.-C., et al., Small molecules as structural and functional mimics of sialyl Lewis X tetrasaccharide in selectin inhibition: a remarkable enhancement of inhibition by additional negative charge and/or hydrophobic group, *J. Am. Chem. Soc.*, 119, 8152–8158, 1997.
43. Zhu, W. and Li, Z., Synthesis of perfluoroalkylated sugars catalyzed by rabbit muscle aldolase (RAMA), *J. Chem. Soc. Perkin Trans. I*, 1105–1108, 2000.
44. Bednarski, M.D., Waldmann, H.J., and Whitesides, G.M., Aldolase-catalyzed synthesis of complex C8 and C9 monosaccharides, *Tetrahedron Lett.*, 27, 5807–5810, 1986.
45. Eyrisch, O. and Fessner, W.D., Disaccharide mimetics by enzymatic tandem aldol additions, *Angew. Chem. Int. Ed. Engl.*, 34, 1639–1641, 1995.
46. Petersen, M., Zannetti, M.T., and Fessner, W.-D., Tandem asymmetric C–C bond formations by enzyme catalysis, *Top. Curr. Chem.*, 186, 87–117, 1997.
47. Fessner, W.-D., Gosse, C., Jaeschke, G., et al., Enzymes in organic synthesis, 15. Short enzymatic synthesis of L-fucose analogs, *Eur. J. Org. Chem.*, 125–132, 2000.
48. Wong, C.-H., Alajarin, R., Moris-Varas, F., et al., Enzymatic synthesis of L-fucose and analogs, *J. Org. Chem.*, 60, 7360–7363, 1995.
49. Durrwachter, J.R., Drueckhammer, D.G., Nozaki, K., et al., Enzymic aldol condensation/isomerization as a route to unusual sugar derivatives, *J. Am. Chem. Soc.*, 108, 7812–7818, 1986.
50. Borysenko, C.W., Spaltenstein, A., Straub, J.A., et al., The synthesis of aldose sugars from half-protected dialdehydes using rabbit muscle aldolase, *J. Am. Chem. Soc.*, 111, 9275–9276, 1989.
51. Look, G.C., Fotsch, C.H., and Wong, C.-H., Enzyme-catalyzed organic synthesis—practical routes to aza sugars and their analogs for use as glycoprocessing inhibitors, *Acc. Chem. Res.*, 26, 182–190, 1993.
52. Kajimoto, T., Chen, L., Liu, K.K.C., et al., Palladium-mediated stereocontrolled reductive amination of azido sugars prepared from enzymic aldol condensation: a general approach to the synthesis of deoxy aza sugars, *J. Am. Chem. Soc.*, 113, 6678–6680, 1991.
53. Ziegler, T., Straub, A., and Effenberger, F., Enzym-katalysierte Synthese von 1-Desoxymannojirimycin, 1-Desoxynojirimycin und 1,4-Dideoxy-1,4-Imino-D-Arabinol, *Angew. Chem. Int. Ed. Engl.*, 27, 716–717, 1988.
54. Liu, K.K.C., Kajimoto, T., Chen, L., et al., Use of dihydroxyacetone phosphate-dependent aldolases in the synthesis of deoxy aza sugars, *J. Org. Chem.*, 56, 6280–6289, 1991.
55. Lees, W.J. and Whitesides, G.M., The enzymatic synthesis of 1,5-dideoxy-1,5-diimino-D-talitol and 1-deoxygalactostatin using fuculose-1-phosphate aldolase, *Bioorg. Chem.*, 20, 173–179, 1992.
56. Zhou, P.Z., Salleh, H.M., Chan, P.C.M., et al., A chemoenzymic synthesis of 1,5-dideoxy-1,5-imino-L-mannitol and 1,5-dideoxy-1,5-imino-L-rhamnitol and investigation of their effects on glycosidases, *Carbohydr. Res.*, 239, 155–166, 1993.
57. Kajimoto, T., Liu, K.K.C., Pederson, R.L., et al., Enzyme-catalyzed aldol condensation for asymmetric synthesis of azasugars: synthesis, evaluation, and modeling of glycosidase inhibitors, *J. Am. Chem. Soc.*, 113, 6187–6196, 1991.
58. Romero, A. and Wong, C.-H., Chemo-enzymatic total synthesis of 3-epiaustraline, australine, and 7-epialexine, *J. Org. Chem.*, 65, 8264–8268, 2000.
59. Schultz, M., Waldmann, H., Kunz, H., et al., Chemoenzymatische “Chiral-Pool”-Synthese von (+)-exo-Brevicomin aus Kohlenhydraten mit Fructose-1,6-diphosphat-Aldolase, *Liebigs Ann. Chem.*, 1019–1024, 1990.
60. Myles, D.C., Andrulis, P.J.I., and Whitesides, G.M., A transketolase-based synthesis of (+)-exo-brevicomin, *Tetrahedron Lett.*, 32, 4835–4838, 1991.

61. Chenevert, R. and Dasser, M., Chemoenzymatic synthesis of the microbial elicitor (–)-syringolide via a fructose 1,6-diphosphate aldolase-catalyzed condensation reaction, *J. Org. Chem.*, 65, 4529–4531, 2000.
62. Chenevert, R., Lavoie, M., and Dasser, M., Use of aldolases in the synthesis of non-carbohydrate natural products. Stereoselective synthesis of aspiciin C-3-C-9 fragment, *Can. J. Chem.*, 75, 68–73, 1997.
63. Shimagaki, M., Muneshima, H., Kubota, M., et al., Chemoenzymatic carbon–carbon bond formation leading to non-carbohydrate derivative—stereoselective synthesis of pentamycin C-11-C-16 fragment, *Chem. Pharm. Bull.*, 41, 282–286, 1993.
64. Phung, A.N., Zannetti, M.T., Whited, G., et al., Stereospecific biocatalytic synthesis of pancratisatin analogues, *Angew. Chem. Int. Ed. Engl.*, 42, 4821–4824, 2003.
65. Colbran, R.L., Jones, J.K.N., Matheson, N.K., et al., A synthesis of dihydroxyacetone phosphate from dihydroxyacetone, *Carbohydr. Res.*, 4, 355–358, 1967.
66. Effenberger, F. and Straub, A., A novel convenient preparation of dihydroxyacetone phosphate and its use in enzymatic aldol reactions, *Tetrahedron Lett.*, 28, 1641–1644, 1987.
67. Jung, S.H., Jeong, J.H., Miller, P., et al., An efficient multigram-scale preparation of dihydroxyacetone phosphate, *J. Org. Chem.*, 59, 7182–7184, 1994.
68. Pederson, R.L., Esker, J., and Wong, C.-H., An improved synthesis of dihydroxyacetone phosphate, *Tetrahedron*, 47, 2643–2648, 1991.
69. Valentin, M.L. and Bolte, J., A convenient synthesis of dihydroxyacetone phosphate from acetone, *Bull. Soc. Chim. Fr.*, 132, 1167–1171, 1995.
70. Gefflaut, T., Lemaire, M., Valentin, M.L., et al., A novel efficient synthesis of dihydroxyacetone phosphate and bromoacetol phosphate for use in enzymatic aldol syntheses, *J. Org. Chem.*, 62, 5920–5922, 1997.
71. Ferroni, E.L., DiTella, V., Ghanayem, N., et al., A three-step preparation of dihydroxyacetone phosphate dimethyl acetal, *J. Org. Chem.*, 64, 4943–4945, 1999.
72. Charmantray, F., El Blidi, L., Gefflaut, T., et al., Improved straightforward chemical synthesis of dihydroxyacetone phosphate through enzymatic desymmetrization of 2,2-dimethoxypropane-1,3-diol, *J. Org. Chem.*, 69, 9310–9312, 2004.
73. Crans, D.C., Kazlauskas, R.J., Hirschbein, B.L., et al., Enzymic regeneration of adenosine 5'-triphosphate: acetyl phosphate, phosphoenolpyruvate, methoxycarbonyl phosphate, dihydroxyacetone phosphate, 5-phospho- α -D-ribosyl pyrophosphate, uridine-5'-diphosphoglucose, *Meth. Enzymol.*, 136, 263–280, 1987.
74. Crans, D.C. and Whitesides, G.M., Glycerol kinase: synthesis of dihydroxyacetone phosphate, sn-glycerol-3-phosphate, and chiral analogs, *J. Am. Chem. Soc.*, 107, 7019–7027, 1985.
75. Sanchez-Moreno, I., Francisco G.-G.J., Bastida, A., et al., Multienzyme system for dihydroxyacetone phosphate-dependent aldolase catalyzed C–C bond formation from dihydroxyacetone, *Chem. Commun.*, 1634–1635, 2004.
76. D'Arrigo, P., Piergianni, V., Pedrocchi-Fantoni, G., et al., Indirect enzymatic phosphorylation: preparation of dihydroxyacetone phosphate, *J. Chem. Soc. Chem. Commun.*, 2505–2506, 1995.
77. Fessner, W.-D. and Sinerius, G., Phosphoenolpyruvate as a dual purpose reagent for integrated nucleotide/nicotinamide cofactor recycling, *Bioorg. Med. Chem.*, 2, 639–645, 1994.
78. Fessner, W.-D. and Sinerius, G., Synthesis of dihydroxyacetone phosphate (and isosteric analogues) by enzymatic oxidation: sugars from glycerol, *Angew. Chem. Int. Ed. Engl.*, 33, 209–212, 1994.
79. Eyrisch, O., Keller, M., and Fessner, W.-D., Higher-carbon sugars by enzymatic chain extension. Oxidative generation of aldol precursors *in situ*, *Tetrahedron Lett.*, 35, 9013–9016, 1994.
80. Espelt, L., Parella, T., Bujons, J., et al., Stereoselective aldol additions catalyzed by dihydroxyacetone phosphate-dependent aldolases in emulsion systems: preparation and structural characterization of linear and cyclic iminopolyols from aminoaldehydes, *Chemistry. Eur. J.*, 9, 4887–4899, 2003.
81. Espelt, L., Clapes, P., Esquena, J., et al., Enzymatic carbon–carbon bond formation in water-in-oil highly concentrated emulsions (gel emulsions), *Langmuir*, 19, 1337–1346, 2003.

82. Schoevaart, R., van Rantwijk, F., and Sheldon, R.A., A four-step enzymatic cascade for the one-pot synthesis of non-natural carbohydrates from glycerol, *J. Org. Chem.*, 65, 6940–6943, 2000.
83. Schoevaart, R., van Rantwijk, F., and Sheldon, R.A., Facile enzymatic aldol reactions with dihydroxyacetone in the presence of arsenate, *J. Org. Chem.*, 66, 4559–4562, 2001.
84. Drueckhammer, D.G., Durrwachter, J.R., Pederson, R.L., et al., Reversible and *in situ* formation of organic arsenates and vanadates as organic phosphate mimics in enzymatic reactions: mechanistic investigation of aldol reactions and synthetic applications, *J. Org. Chem.*, 54, 70–77, 1989.
85. Stribling, D., Properties of the phosponomethyl isosteres of two phosphate ester glycolytic intermediates, *Biochem. J.*, 141, 725–728, 1974.
86. Arth, H.L. and Fessner, W.-D., Practical synthesis of 4-hydroxy-3-oxobutylphosphonic acid and its evaluation as a bio-isosteric substrate of DHAP aldolase, *Carbohydr. Res.*, 305, 313–321, 1997.
87. Duncan, R. and Drueckhammer, D.G., Preparation of deoxy sugars via aldolase-catalyzed synthesis of 1-deoxy-1-thioketoses, *J. Org. Chem.*, 61, 438–439, 1996.
88. Raetz, C.R.H. and Dowhan, W., Biosynthesis and function of phospholipids in *Escherichia coli*, *J. Biol. Chem.*, 265, 1235–1238, 1990.
89. Varki, A., Diversity in the sialic acids, *Glycobiology*, 2, 25–40, 1992.
90. Varki, A., Biological roles of oligosaccharides—all of the theories are correct, *Glycobiology*, 3, 97–130, 1993.
91. Fessner, W.-D. and Knorst, M., Manufacture of the *N*-acetylneuraminic acid synthase of *Neisseria meningitidis* for synthesis of modified sialic acids expression of the cloned gene, DE10034586: 2002 (*Chem. Abstr.* 2002, 136, 166160).
92. Barbosa, J.A., Smith, B.J., DeGori, R., et al., Active site modulation in the *N*-acetylneuraminylase sub-family as revealed by the structure of the inhibitor-complexed *Haemophilus influenzae* enzyme, *J. Mol. Biol.*, 303, 405–421, 2000.
93. Kim, M.J., Hennen, W.J., Sweers, H.M., et al., Enzymes in carbohydrate synthesis: *N*-acetylneuraminic acid aldolase catalyzed reactions and preparation of *N*-acetyl-2-deoxy-D-neuraminic acid derivatives, *J. Am. Chem. Soc.*, 110, 6481–6486, 1988.
94. Sugai, T., Shen, G.J., Ichikawa, Y., et al., Synthesis of 3-deoxy-D-manno-2-octulosonic acid (KDO) and its analogs based on KDO aldolase-catalyzed reactions, *J. Am. Chem. Soc.*, 115, 413–421, 1993.
95. Allen, S.T., Heintzelman, G.R., and Toone, E.J., Pyruvate aldolases as reagents for stereospecific aldol condensation, *J. Org. Chem.*, 57, 426–427, 1992.
96. Shelton, M.C., Cotterill, I.C., Novak, S.T.A., et al., 2-Keto-3-deoxy-6-phosphogluconate aldolases as catalysts for stereocontrolled carbon–carbon bond formation, *J. Am. Chem. Soc.*, 118, 2117–2125, 1996.
97. Cotterill, I.C., Shelton, M.C., Machemer, D.E.W., et al., Effect of phosphorylation on the reaction rate of unnatural electrophiles with 2-keto-3-deoxy-6-phosphogluconate aldolase, *J. Chem. Soc. Perkin Trans. I*, 1335–1341, 1998.
98. Beliczey, J., Kragl, U., Liese, A., et al., Method for making fluorinated sugars having a side chain and use thereof, US 6355453: 2002 (*Chem. Abstr.* 2002, 136, 231340).
99. Kragl, U., Gödde, A., Wandrey, C., et al., New synthetic applications of sialic acid aldolase, a useful catalyst for KDO synthesis—relation between substrate conformation and enzyme stereoselectivity, *J. Chem. Soc. Perkin Trans. I*, 119–124, 1994.
100. Fitz, W., Schwark, J.R., and Wong, C.H., Aldotetroses and C(3)-modified aldohexoses as substrates for *N*-acetylneuraminic acid aldolase: a model for the explanation of the normal and the inversed stereoselectivity, *J. Org. Chem.*, 60, 3663–3670, 1995.
101. Augé, C., Gautheron, C., David, S., et al., Sialyl aldolase in organic synthesis: from the trout egg acid, 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), to branched-chain higher ketoses as possible new chirons, *Tetrahedron*, 46, 201–214, 1990.
102. Liu, J.L.C., Shen, G.J., Ichikawa, Y., et al., Overproduction of CMP-sialic acid synthetase for organic synthesis, *J. Am. Chem. Soc.*, 114, 3901–3910, 1992.
103. Lin, C.-C., Lin, C.-H., and Wong, C.-H., Sialic acid aldolase-catalyzed condensation of pyruvate and *N*-substituted mannosamine: a useful method for the synthesis of *N*-substituted sialic acids, *Tetrahedron Lett.*, 38, 2649–2652, 1997.

104. Wu, W.-Y., Jin, B., Kong, D.C.M., et al., A facile synthesis of a useful 5-*N*-substituted-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid from 2-acetamido-2-deoxy-D-glucose, *Carbohydr. Res.*, 300, 171–174, 1997.
105. Kuboki, A., Okazaki, H., Sugai, T., et al., An expeditions route to *N*-glycolylneuraminic acid based on enzyme-catalyzed reaction, *Tetrahedron*, 53, 2387–2400, 1997.
106. Humphrey, A.J., Fremann, C., Critchley, P., et al., Biological properties of *N*-acyl and *N*-haloacetyl neuraminic acids: processing by enzymes of sialic acid metabolism, and interaction with influenza virus, *Bioorg. Med. Chem.*, 10, 3175–3185, 2002.
107. Pan, Y., Ayani, T., Nadas, J., et al., Accessibility of *N*-acyl-D-mannosamines to *N*-acetyl-D-neuraminic acid aldolase, *Carbohydr. Res.*, 339, 2091–2100, 2004.
108. Murakami, M., Ikeda, K., and Achiwa, K., Chemoenzymatic synthesis of neuraminic acid analogs structurally varied at C-5 and C-9 as potential inhibitors of the sialidase from influenza virus, *Carbohydr. Res.*, 280, 101–110, 1996.
109. Kong, D.C.M. and von Itzstein, M., The chemoenzymatic synthesis of 9-substituted 3,9-dideoxy-D-glycero-D-galacto-2-nonulosonic acids, *Carbohydr. Res.*, 305, 323–329, 1997.
110. Kiefel, M.J., Wilson, J.C., Bennett, S., et al., Synthesis and evaluation of C-9 modified *N*-acetylneuraminic acid derivatives as substrates for *N*-acetylneuraminic acid aldolase, *Bioorg. Med. Chem.*, 8, 657–664, 2000.
111. Kok, G.B., Campbell, M., Mackey, B.L., et al., Synthesis of C-3 nitrogen-containing derivatives of *N*-acetyl- α , β -D-mannosamine as substrates for *N*-acetylneuraminic acid aldolase, *Carbohydr. Res.*, 332, 133–139, 2001.
112. Lins, R.J., Flitsch, S.L., Turner, N.J., et al., Generation of a dynamic combinatorial library using sialic acid aldolase and *in situ* screening against wheat germ agglutinin, *Tetrahedron*, 60, 771–780, 2004.
113. Lin, C.H., Sugai, T., Halcomb, R.L., et al., Unusual stereoselectivity in sialic acid aldolase-catalyzed aldol condensations—synthesis of both enantiomers of high-carbon monosaccharides, *J. Am. Chem. Soc.*, 114, 10138–10145, 1992.
114. David, S., Malleron, A., and Cavaye, B., Aldolases in organic synthesis: acylneuraminate-pyruvate lyase accepts furanoses as substrates, *New J. Chem.*, 16, 751–755, 1992.
115. Koppert, K. and Brossmer, R., Synthesis of the C-5 homologue of *N*-acetylneuraminic acid by enzymatic chain elongation of 2-C-acetamidomethyl-2-deoxy-D-mannose, *Tetrahedron Lett.*, 33, 8031–8034, 1992.
116. Malleron, A. and David, S., An enzymic approach to sequence 12–20 of Amphotericin B, *New J. Chem.*, 20, 153–159, 1996.
117. Zhou, P.Z., Salleh, H.M., and Honek, J.F., Facile chemoenzymatic synthesis of 3-(hydroxymethyl)-6-epicastanospermine, *J. Org. Chem.*, 58, 264–266, 1993.
118. Henderson, D.P., Shelton, M.C., Cotterill, I.C., et al., Stereospecific preparation of the N-terminal amino acid moiety of nikkomycins K-X and K-Z via a multiple enzyme synthesis, *J. Org. Chem.*, 62, 7910–7911, 1997.
119. Sugai, T., Kuboki, A., Hiramatsu, S., et al., Improved enzymatic procedure for a preparative-scale synthesis of sialic acid and KDN, *Bull. Chem. Soc. Jpn.*, 68, 3581–3589, 1995.
120. Blayer, S., Woodley, J.M., Lilly, M.D., et al., Characterization of the chemoenzymatic synthesis of *N*-acetyl-D-neuraminic acid (Neu5Ac), *Biotechnol. Prog.*, 12, 758–763, 1996.
121. Mahmoudian, M., Noble, D., Drake, C.S., et al., An efficient process for production of *N*-acetylneuraminic acid using *N*-acetylneuraminic acid aldolase, *Enzyme Microb. Technol.*, 20, 393–400, 1997.
122. Kragl, U., Gyga, D., Ghisalba, O., et al., Enzymatic, two-step synthesis of *N*-acetylneuraminic acid in a enzyme-membrane reactor, *Angew. Chem. Int. Ed. Engl.*, 30, 827–828, 1991.
123. Maru, I., Ohnishi, J., Ohta, Y., et al., Simple and large-scale production of *N*-acetylneuraminic acid from *N*-acetyl-D-glucosamine and pyruvate using *N*-acyl-D-glucosamine 2-epimerase and *N*-acetylneuraminate lyase, *Carbohydr. Res.*, 306, 575–578, 1998.
124. Lee, J.-O., Yi, J.-K., Lee, S.-G., et al., Production of *N*-acetylneuraminic acid from *N*-acetylglucosamine and pyruvate using recombinant human renin binding protein and sialic acid aldolase in one pot, *Enzyme Microb. Technol.*, 35, 121–125, 2004.

125. Salagnad, C., Godde, A., Ernst, B., et al., Enzymatic large-scale production of 2-keto-3-deoxy-D-glycero-D-galacto-nonopyranulosonic acid in enzyme membrane reactors, *Biotechnol. Prog.*, 13, 810–813, 1997.
126. Ichikawa, Y., Liu, J.L.C., Shen, G.J., et al., A highly efficient multienzyme system for the one-step synthesis of a sialyl trisaccharide: *in situ* generation of sialic acid and N-acetylglucosamine coupled with regeneration of UDP-glucose, UDP-galactose and CMP-sialic acid, *J. Am. Chem. Soc.*, 113, 6300–6302, 1991.
127. Ichikawa, Y., Wang, R., and Wong, C.H., Regeneration of sugar nucleotide for enzymatic oligosaccharide synthesis, *Meth. Enzymol.*, 247, 107–127, 1994.
128. Blixt, O. and Paulson, J.C., Biocatalytic preparation of N-glycolylneuraminic acid, deaminoneuraminic acid (KDN) and 9-azido-9-deoxysialic acid oligosaccharides, *Adv. Synth. Catal.*, 345, 687–690, 2003.
129. Yu, H., Yu, H., Karpel, R., et al., Chemoenzymatic synthesis of CMP-sialic acid derivatives by a one-pot two-enzyme system: comparison of substrate flexibility of three microbial CMP-sialic acid synthetases, *Bioorg. Med. Chem.*, 12, 6427–6435, 2004.
130. Knorst, M. and Fessner, W.-D., CMP-sialate synthetase from *Neisseria meningitidis*—overexpression and application to the synthesis of oligosaccharides containing modified sialic acids, *Adv. Synth. Catal.*, 343, 698–710, 2001.
131. Heine, A., Luz, J.G., Wong, C.-H., et al., Analysis of the class I aldolase binding site architecture based on the crystal structure of 2-deoxyribose-5-phosphate aldolase at 0.99 Å resolution, *J. Mol. Biol.*, 343, 1019–1034, 2004.
132. Barbas, C.F., Wang, Y.F., and Wong, C.-H., Deoxyribose-5-phosphate aldolase as a synthetic catalyst, *J. Am. Chem. Soc.*, 112, 2013–2014, 1990.
133. Chen, L., Dumas, D.P., and Wong, C.-H., Deoxyribose-5-phosphate aldolase as a catalyst in asymmetric aldol condensation, *J. Am. Chem. Soc.*, 114, 741–748, 1992.
134. Wong, C.-H., Garcia-Junceda, E., Chen, L.R., et al., Recombinant 2-deoxyribose-5-phosphate aldolase in organic synthesis: use of sequential two-substrate and three-substrate aldol reactions, *J. Am. Chem. Soc.*, 117, 3333–3339, 1995.
135. Liu, J. and Wong, C.-H., Aldolase-catalyzed asymmetric synthesis of novel pyranose synthons as a new entry to heterocycles and epothilones, *Angew. Chem. Int. Ed. Engl.*, 41, 1404–1407, 2002.
136. DeSantis, G., Liu, J., Clark, D.P., et al., Structure-based mutagenesis approaches toward expanding the substrate specificity of D-2-deoxyribose-5-phosphate aldolase, *Bioorg. Med. Chem.*, 11, 43–52, 2003.
137. Ouwerkerk, N., Van Boom, J.H., Lugtenburg, J., et al., Chemo-enzymatic synthesis of thymidine 13C-labeled in the 2'-Deoxyribose moiety, *Eur. J. Org. Chem.*, 861–866, 2000.
138. Tischer, W., Ihlenfeldt, H.-G., Barzu, O., et al., Enzymatic synthesis of deoxyribonucleosides from deoxyribose 1-phosphate and nucleobase, PCT WO0114566: 2001 (*Chem. Abstr.* 2001, 134, 208062).
139. Horinouchi, N., Ogawa, J., Sakai, T., et al., Construction of deoxyriboaldolase-overexpressing *Escherichia coli* and its application to 2-deoxyribose 5-phosphate synthesis from glucose and acetaldehyde for 2'-deoxyribonucleoside production, *Appl. Environ. Microbiol.*, 69, 3791–3797, 2003.
140. Gijzen, H.J.M. and Wong, C.-H., Unprecedented asymmetric aldol reactions with three aldehyde substrates catalyzed by 2-deoxyribose-5-phosphate aldolase, *J. Am. Chem. Soc.*, 116, 8422–8423, 1994.
141. Liu, J., Hsu, C.-C., and Wong, C.-H., Sequential aldol condensation catalyzed by DERA mutant Ser238Asp and a formal total synthesis of atorvastatin, *Tetrahedron Lett.*, 45, 2439–2441, 2004.
142. Greenberg, W.A., Varvak, A., Hanson, S.R., et al., Development of an efficient, scalable, aldolase-catalyzed process for enantioselective synthesis of statin intermediates, *Proc. Natl. Acad. Sci. USA*, 101, 5788–5793, 2004.
143. Kierkels, J.G.T., Mink, D., Panke, S., et al., Process for the preparation of 2,4-dideoxyhexoses and 2,4,6-trideoxyhexoses and therapeutic uses thereof, WO0306656: 2003 (*Chem. Abstr.* 2003, 138, 112523).
144. Müller, M. Chemoenzymatic synthesis of building blocks for statin side chains, *Angew. Chem. Int. Ed. Engl.*, 44, 362–365, 2005.

145. Liu, J.Q., Dai, T., Itoh, N., et al., Diversity of microbial threonine aldolases and their application, *J. Mol. Catal., B Enzym.*, 10, 107–115, 2000.
146. Kimura, T., Vassilev, V.P., Shen, G.J., et al., Enzymatic synthesis of beta-hydroxy-alpha-amino acids based on recombinant D- and L-threonine aldolases, *J. Am. Chem. Soc.*, 119, 11734–11742, 1997.
147. Saeed, A. and Young, D.W., Synthesis of L-β-hydroxy amino acids using serine hydroxymethyltransferase, *Tetrahedron*, 48, 2507–2514, 1992.
148. Lotz, B.T., Gasparski, C.M., Peterson, K., et al., Substrate specificity studies of aldolase enzymes for use in organic synthesis, *J. Chem. Soc. Chem. Commun.*, 16, 1107–1109, 1990.
149. Vassilev, V.P., Uchiyama, T., Kajimoto, T., et al., L-Threonine aldolase in organic synthesis: preparation of novel beta-hydroxy-alpha-amino acids, *Tetrahedron Lett.*, 36, 4081–4084, 1995.
150. Genet, J.-P., New asymmetric syntheses of β-hydroxy α-amino acids and analogs. Components of biologically active cyclopeptides, *Pure Appl. Chem.*, 68, 593–596, 1996.
151. Hsiao, H.-Y. and Wei, T., Enzymatic production of L-serine with a feedback control system for formaldehyde addition, *Biotechnol. Bioeng.*, 28, 1510–1518, 1986.
152. Hsiao, H.-Y., Wei, T., and Campbell, K., Enzymatic production of L-serine, *Biotechnol. Bioeng.*, 28, 857–867, 1986.
153. Ura, D., Hashimukai, T., Matsumoto, T., et al., Enzymic preparation of L-serine, EP 421477: 1991 (*Chem. Abstr.* 1991, 115, 90657).
154. Anderson, D.M. and Hsiao, H.-H., Enzymatic method for L-serine production, in *Biocatalytic Production of Amino Acids and Derivatives*, Rozzell, D. and Wagner, F., Eds., Hanser, Munich, 1992, pp. 23–41.
155. Bycroft, M., Herbert, R.B., and Ellames, G.J., Heterocyclic β-hydroxy-α-amino acids as substrates for a novel aldolase from *Streptomyces amakusaensis*; preparation of (2R,3R)-3-(2-thienyl)serine and (2R,3R)-3-(2-furyl)serine from racemic threo material, *J. Chem. Soc. Perkin Trans. I*, 2439–2442, 1996.
156. Fujii, M., Miura, T., Kajimoto, T., et al., Facile synthesis of 3,4-dihydroxyprolines as an application of the L-threonine aldolase-catalyzed aldol reaction, *Synlett*, 1046–1048, 2000.
157. Shibata, K., Shingu, K., Vassilev, V.P., et al., Kinetic and thermodynamic control of L-threonine aldolase catalyzed reaction and its application to the synthesis of mycetericin D, *Tetrahedron Lett.*, 37, 2791–2794, 1996.
158. Morikawa, T., Ikemi, M., and Miyoshi, T., Manufacture of D-erythro-3-(3,4-dihydroxyphenyl)serine derivatives with microorganisms or enzymes, 1992 (*Chem. Abstr.* 1992, 118, 190107).
159. Morikawa, T., Ikemi, M., and Miyoshi, T., D-Erythro-hydroxyamino acids enzymic preparation, 1994 (*Chem. Abstr.* 1994, 121, 155936).
160. Herbert, R.B., Wilkinson, B., and Ellames, G.J., Preparation of (2R,3S)-beta-hydroxy-alpha-amino acids by use of a novel streptomyces aldolase as a resolving agent for racemic material, *Can. J. Chem.*, 72, 114–117, 1994.
161. Liu, J.Q., Odani, M., Dai, T., et al., A new route to L-threo-3-[4-(methylthio)phenylserine], a key intermediate for the synthesis of antibiotics: recombinant low-specificity D-threonine aldolase-catalyzed stereospecific resolution, *Appl. Microbiol. Biotechnol.*, 51, 586–591, 1999.
162. Liu, J.Q., Odani, M., Yasuoka, T., et al., Gene cloning and overproduction of low-specificity D-threonine aldolase from *Alcaligenes xylosoxidans* and its application for production of a key intermediate for parkinsonism drug, *Appl. Microbiol. Biotechnol.*, 54, 44–51, 2000.
163. Schörken, U. and Sprenger, G.A., Thiamin-dependent enzymes as catalysts in chemoenzymatic syntheses, *Biochim. Biophys. Acta*, 1385, 229–243, 1998.
164. Zimmermann, F.T., Schneider, A., Schörken, U., et al., Efficient multi-enzymatic synthesis of D-xylulose 5-phosphate, *Tetrahedron Asymmetry*, 10, 1643–1646, 1999.
165. Turner, N.J., Applications of transketolases in organic synthesis, *Curr. Opin. Biotechnol.*, 11, 527–531, 2000.
166. Bolte, J., Demuynck, C., and Samaki, H., Utilization of enzymes in organic chemistry: transketolase catalyzed synthesis of ketoses, *Tetrahedron Lett.*, 28, 5525–5528, 1987.
167. Morris, K.G., Smith, M.E.B., Turner, N.J., et al., Transketolase from *Escherichia coli*: a practical procedure for using the biocatalyst for asymmetric carbon-carbon bond synthesis, *Tetrahedron Asymmetry*, 7, 2185–2188, 1996.

168. Kobori, Y., Myles, D.C., and Whitesides, G.M., Substrate specificity and carbohydrate synthesis using transketolase, *J. Org. Chem.*, 57, 5899–5907, 1992.
169. Effenberger, F., Null, V., and Ziegler, T., Enzyme catalyzed reactions. 12. Preparation of optically pure L-2-hydroxyaldehydes with yeast transketolase, *Tetrahedron Lett.*, 33, 5157–5160, 1992.
170. French, C. and Ward, J.M., Improved production and stability of *E. coli* recombinants expressing transketolase for large scale biotransformation, *Biotechnol. Lett.*, 17, 247–252, 1995.
171. Hobbs, G.R., Mitra, R.K., Chauhan, R.P., et al., Enzyme-catalysed carbon–carbon bond formation: large-scale production of *Escherichia coli* transketolase, *J. Biotechnol.*, 45, 173–179, 1996.
172. Bongs, J., Hahn, D., Schörken, U., et al., Continuous production of erythrulose using transketolase in a membrane reactor, *Biotechnol. Lett.*, 19, 213–215, 1997.
173. Brocklebank, S., Woodley, J.M., and Lilly, M.D., Immobilised transketolase for carbon–carbon bond synthesis: biocatalyst stability, *J. Mol. Catal., B Enzym.*, 7, 223–231, 1999.
174. Chauhan, R.P., Woodley, J.M., and Powell, L.W., *In situ* product removal from *E. coli* transketolase-catalyzed biotransformations, *Ann. N. Y. Acad. Sci.*, 799, 545–554, 1996.
175. Effenberger, F. and Null, V., Enzyme-catalyzed reactions. 13. A new, efficient synthesis of fagomine, *Liebigs Ann. Chem.*, 1211–1212, 1992.
176. Humphrey, A.J., Parsons, S.F., Smith, M.E.B., et al., Synthesis of a novel *N*-hydroxypyrrolidine using enzyme catalysed asymmetric carbon–carbon bond synthesis, *Tetrahedron Lett.*, 41, 4481–4485, 2000.
177. Gonzalez-Garcia, E., Helaine, V., Klein, G., et al., Fluorogenic stereochemical probes for transaldolases, *Chemistry Eur. J.*, 9, 893–899, 2003.
178. Machajewski, T.D. and Wong, C.-H., The catalytic asymmetric aldol reaction, *Angew. Chem. Int. Ed. Engl.*, 39, 1352–1374, 2000.
179. Palomo, C., Oiarbide, M., and Garcia, J.M., Current progress in the asymmetric aldol addition reaction, *Chem. Soc. Rev.*, 33, 65–75, 2004.
180. Kazmeier, U., Amino acids—valuable organocatalysts in carbohydrate synthesis, *Angew. Chem. Int. Ed. Engl.*, 44, 2186–2188, 2005.
181. Powell, K.A., Ramer, S.W., del Cardayré, S.B., et al., Directed evolution and biocatalysis, *Angew. Chem. Int. Ed. Engl.*, 40, 3948–3959, 2001.
182. Williams, G.J., Nelson, A.S., and Berry, A., Directed evolution of enzymes for biocatalysis and the life sciences, *Cell. Mol. Life Sci.*, 61, 3034–3046, 2004.
183. Tanaka, F., Fuller, R., Shim, H., et al., Evolution of aldolase antibodies in vitro: correlation of catalytic activity and reaction-based selection, *J. Mol. Biol.*, 335, 1007–1018, 2004.
184. Zhu, X., Tanaka, F., Hu, Y., et al., The origin of enantioselectivity in aldolase antibodies: crystal structure, site-directed mutagenesis, and computational analysis, *J. Mol. Biol.*, 343, 1269–1280, 2004.
185. Lorenz, P., Liebeton, K., Niehaus, F., et al., Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space, *Curr. Opin. Biotechnol.*, 13, 572–577, 2002.
186. Streit, W.R., Daniel, R., and Jaeger, K.-E., Prospecting for biocatalysts and drugs in the genomes of non-cultured microorganisms, *Curr. Opin. Biotechnol.*, 15, 285–290, 2004.
187. Wahler, D. and Reymond, J.-L., Novel methods for biocatalyst screening, *Curr. Opin. Chem. Biol.*, 5, 152–158, 2001.

14 Enzymatic Synthesis of Modified Nucleosides

Luís A. Condezo, Jesús Fernández-Lucas, Carlos A. García-Burgos, Andrés R. Alcántara, and José V. Sinisterra

CONTENTS

14.1	Introduction	401
14.2	Enzymatic Synthesis of Modified Nucleosides	402
14.2.1	Enzymes That Modify the Base	402
14.2.2	Enzymes That Modify the Sugar	405
14.2.3	Enzymatic Oxidation of CH ₂ OH in Nucleoside Analogs	407
14.2.4	Enzymes That Catalyze the Synthesis of Nucleosides by Transglycosylation	408
14.2.5	Characterization of the Enzymes Involved in the Biotransformation of <i>N</i> -2'-Deoxyribosyl Nucleosides	410
14.2.6	Immobilization of Whole Cells	411
14.2.7	Synthesis of Different Nucleoside Analogs	414
14.3	Conclusions	420
	References	420

14.1 INTRODUCTION

Nucleosides are involved in many biochemical processes, notably the storage and transfer of genetic information. As a consequence, nucleoside analogs have been used in the treatment of anti-immunodeficiency syndrome (AIDS), and other viral infections, such as those caused by herpes viruses, and influenza A and B viruses [1]. Most of the approved antiviral drugs, such as 3'-azido-2',3'-dideoxythymidine (AZT, **1**) or 2',3'-dideoxyinosine (ddI, **2**), shown in [Figure 14.1](#), are naturally occurring nucleoside analogs that act by interfering with the synthesis of viral nucleic acids.

In addition to the classical antiviral activity of nucleosides, there are some other therapeutic effects described for these nucleoside analogs, such as:

1. Nucleoside transport inhibitors: this strategy is used for cardio-protection and brain protection in ischemic heart disease and stroke, respectively, and in some human leukemia [2]
2. Treatment of inflammatory processes [3]
3. Antisense oligonucleotides, useful for preparing triple ADN helix, as potential and selective inhibitors of gene expression [4]
4. Antimicrobial drugs, such as oxetanocine (AXT-A) or oxanosine [5]

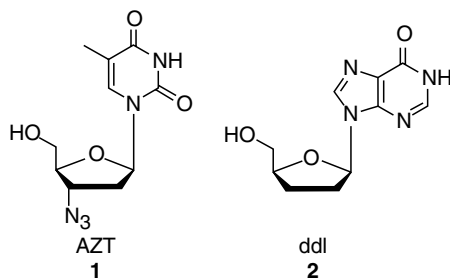


FIGURE 14.1 Approved antiviral drugs: 3'-azido-2',3'-dideoxythymidine (AZT, **1**) or 2',3'-dideoxyinosine (ddI, **2**).

Therefore, much effort has been expended on the synthesis of nucleoside analogs. In this sense, the chemical syntheses of these compounds are often multistage processes, including several protection and deprotection steps in order to obtain the specific modification of certain groups of these polyfunctional molecules [6–11]. On the other hand, the enzymatic synthesis of nucleoside analogs offers several advantages over chemical methods:

1. Protecting groups are not usually required.
2. The enzymatic steps are highly stereospecific and/or regioselective.
3. Enzyme-catalyzed reactions are usually so efficient that the synthesized nucleosides can be used in further processes without purification. Some reviews about this topic have been performed [12,13].

Four different types of enzymes can be used to carry out the chemoenzymatic synthesis of nucleoside analogs:

1. Hydrolytic enzymes capable of modifying functional groups of the base; adenosine deaminase is the most interesting enzyme
2. Hydrolytic enzymes that can modify the functional groups of the sugar residue, such as penicillinacylase, lipases, proteases, and esterases
3. Oxygenases that can oxidize the CH_2OH group of the pentose ring to CO_2H
4. Enzymes that catalyze the transfer of glycosyl residues from a nucleoside donor to an acceptor base. There are two main subclasses of these enzymes:

- Nucleoside phosphorylases
- N-2'-deoxyribosyl transferases

14.2 ENZYMATIC SYNTHESIS OF MODIFIED NUCLEOSIDES

14.2.1 ENZYMES THAT MODIFY THE BASE

Intracellular adenosine deaminase (ADA) is widely used in the preparation of antiviral structures by deamination of 6-aminopurines [14,15]. ADA specifically catalyzes the hydrolytic deamination of 6-aminopurine nucleoside, leaving the amino group of the 2'-amino sugar moiety intact (Figure 14.2).

In addition, this enzyme displays broad substrate specificity and its use can be extended to carbocyclonucleosides or acyclonucleosides [15]. Traditionally, it has been used in the synthesis of guanosine or inosine nucleoside analogs with moderated to high yields (40 to 80%)

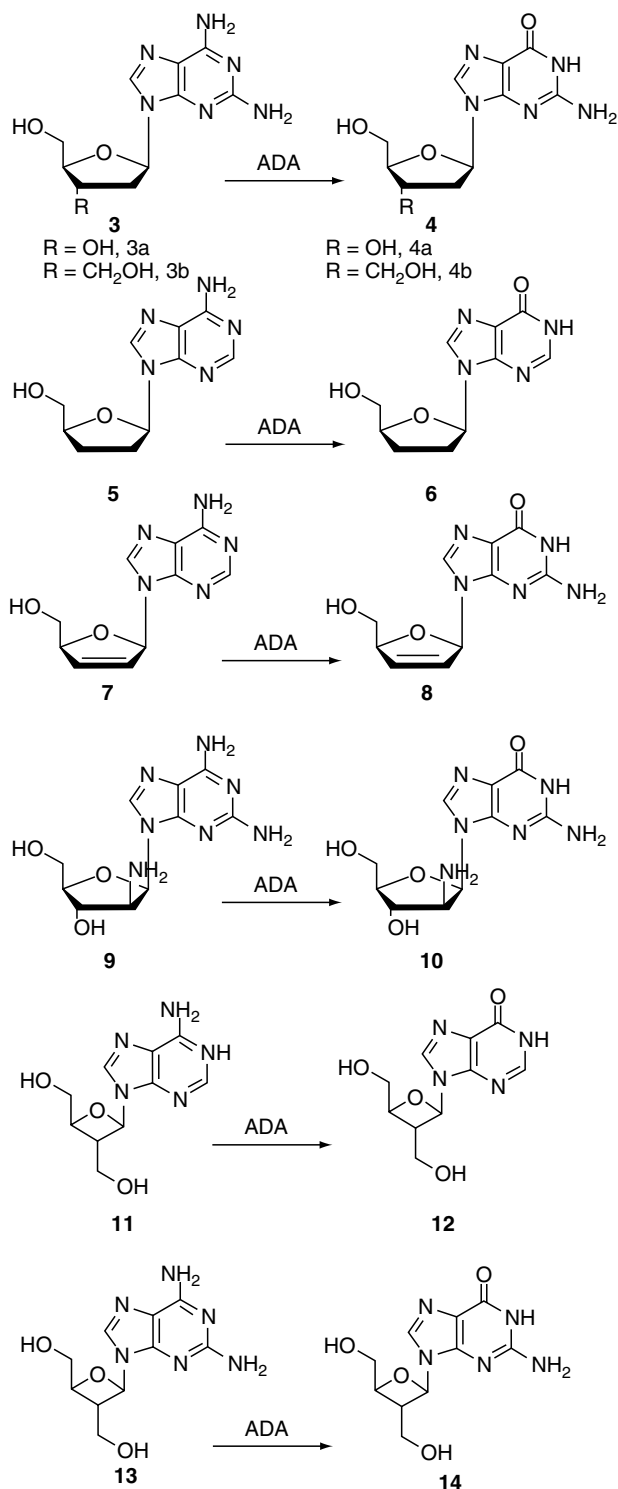


FIGURE 14.2 Applications of adenosine deaminase (ADA) to the synthesis of some nucleoside analogs. (From Rachakonda, S. and Cartee, L., *Curr. Med. Chem.*, 11(6), 775–793, 2004; Ferrero, M. and Gotor, V., *Monatsh. Chem.*, 131(6), 585–616, 2000; Kakefuda, A., Shuto, S., Nagahata, T., et al., *Tetrahedron*, 50(34), 10167–10182, 1994.)

[16], allowing the scalable synthesis of guanosine derivatives. ADA has also been used in the deamination of oxetanocin A (OXT-A, **11**) to the corresponding hypoxanthine (OXT-H, **12**) analog in quantitative yields, or in the specific transformation of 2-amino-OXT-A (**13**) in OXT-G (**14**) (Figure 14.2) [5].

Adenosine deaminase has also been used in the resolution of racemic nucleoside analogs derived from 6-aminopurines. Thus, Hertel et al. [17] reported the preparation of β -1-(2-amino-6-oxo-1*H*,9*H*-purin-9-yl)-2',3'-dideoxy-2',2'-difluororibose (β -**15**) from a racemic mixture of 1-(2,6-diamino-9*H*-purin-9-yl)-2',3'-dideoxy-2',2'-difluororibose (rac-**15**) (Figure 14.3). With the same strategy, the synthesis of both antipodes of the anti-HIV drug carbovir [(-)-**17** and (+)-**17**] has been described [18], starting from racemic *cis*-[3-(2,6)-diamino-9*H*-purin-9-yl]cyclopentyl carbinol (rac-**16**) (Figure 14.3).

Margolin et al. [19] and Santaniello et al. [15] have used adenylic acid deaminase (AMPDA) from *Aspergillus niger* in the synthesis of 6-oxopurine nucleoside analogs with moderated to good yields (40 to 70%). This enzyme shows much broader substrate specificity than ADA, as depicted in Figure 14.4, and can lead to deamination of adenosine derivatives including phosphorylated cyclic (**18**), carbocyclic (**20**), and acyclic analogs [15]. In addition,

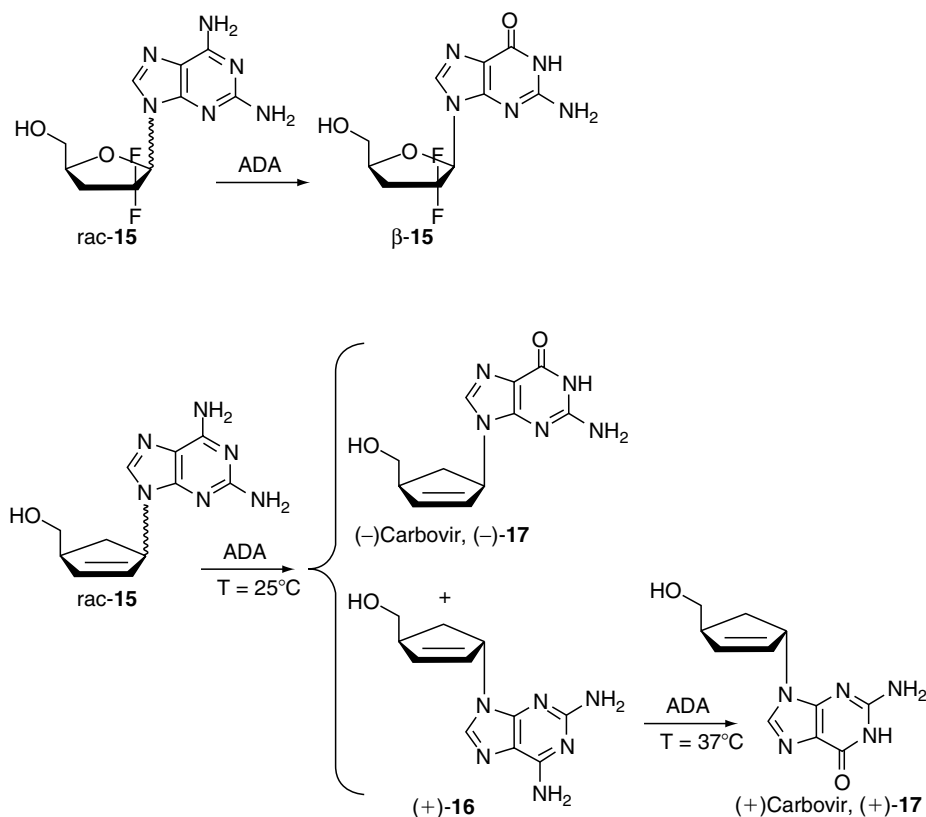


FIGURE 14.3 Resolution of racemic nucleoside analogs using adenosine deaminase (ADA). (From Hertel, L.W., Grossman, C.S., and Kroin, J.S., Eur. Pat. Appl. 0328345, 1989; Vince, R. and Brownell, J., *Biochem. Biophys. p. Res. Co.*, 168(3), 912–916, 1990.)

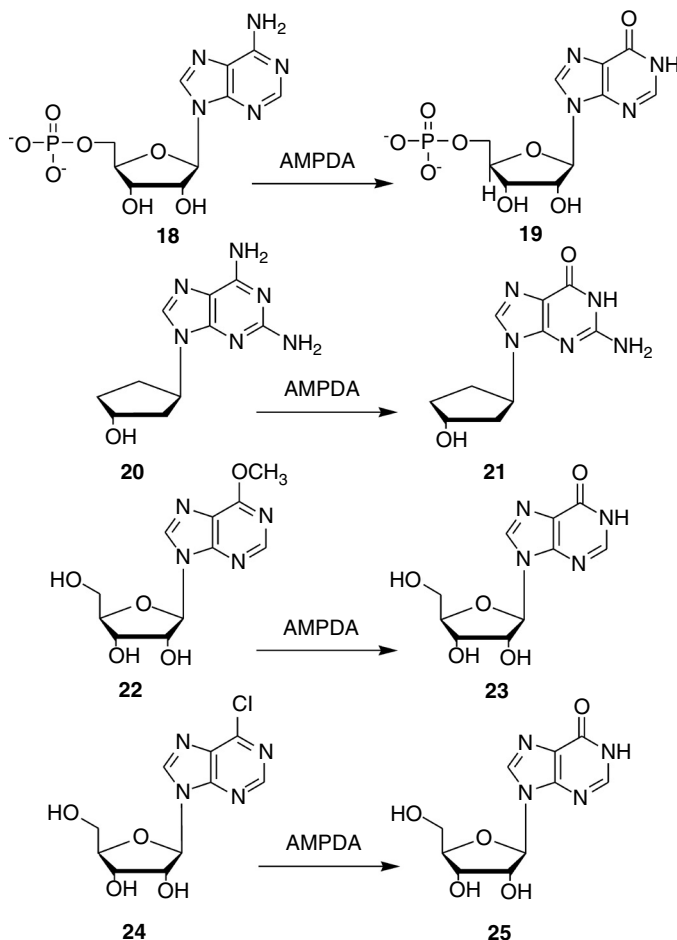


FIGURE 14.4 Synthesis of nucleoside analogs using adenylic acid deaminase (AMPDA). (From Margolin, A.L., Borchering, D.R., Wolf-Kugel, et al., *J. Org. Chem.*, 59(24), 7214–7218, 1994.)

the enzyme can catalyze demethylation (**22**) and dechlorination (**24**) of purine ribonucleosides (Figure 14.4).

14.2.2 ENZYMES THAT MODIFY THE SUGAR

The direct protection–deprotection of hydroxyl groups of nucleosides is a key step in the synthesis of nucleoside analogs. Although several chemical methods are available for regioselective acylation of the nucleoside sugar moiety, enzymatic methods offer advantages considering yield, regioselectivity, and overall number of synthetic steps that have to be carried out. Gotor et al. [20–22] described oxime esters and oxime carbonates as suitable acyl donors in the lipase-catalyzed regioselective acylation of different –OH residues of sugars (Figure 14.5) with good yields. More concretely, *Pseudomonas cepacia* lipase regioselectively acylates in 3'-position when using the oxime carbonate (**27**), while *Candida antarctica* lipase B leads to the acylation exclusively in the 5'-position (**29**) (Figure 14.5). The regioselectivity is only associated to the enzyme rather than to the acyl-donor structure, as it was proved by the same research group [21] using vinyl benzoate and lipase B from *C. antarctica*

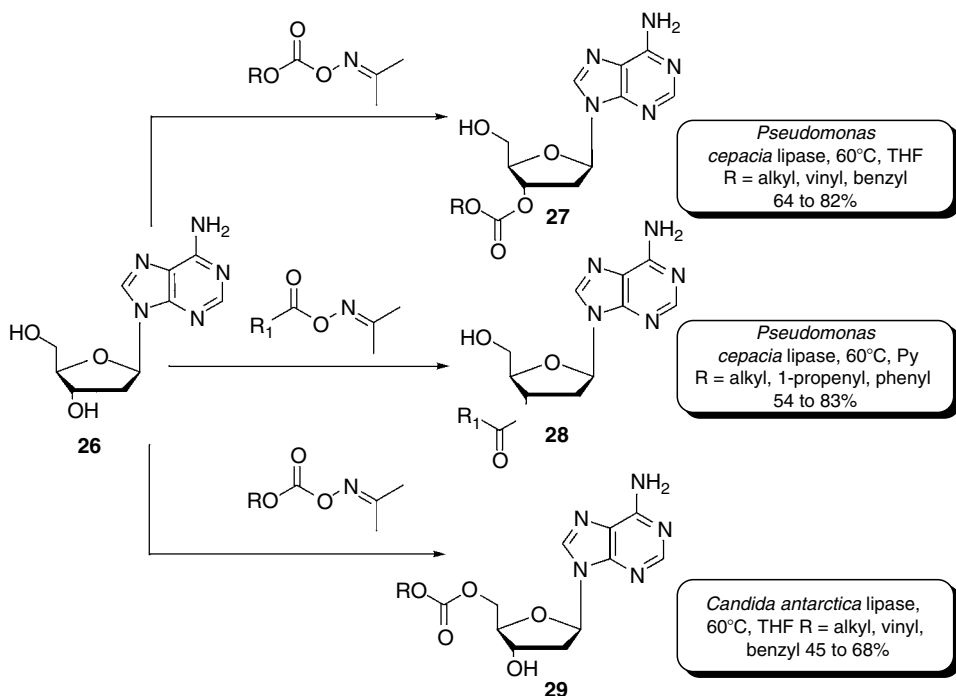


FIGURE 14.5 Regioselective acylation of sugar moiety using oxime esters or oxime carbonates. (From Moris, F. and Gotor, V., *J. Org. Chem.*, 57(8), 2490–2492, 1992; Moris, F. and Gotor, V., *J. Org. Chem.*, 58(3), 653–660, 1993; García, J., Fernández, S., Ferrero, M., et al., *Tetrahedron Lett.*, 45(8), 1709–1712, 2004.)

(28, Figure 14.5). Indeed, 2'-deoxyuridine, thymidine, 2'-deoxyadenosine, and 2'-deoxyguanosine were benzoyleated in 5'-position with moderated yields (13 to 76%), although larger reaction times were necessary to achieve the maximum yields (71 to 80 h).

Subtilisin leads to the same regioselectivity as *P. cepacia* lipase with similar yields, though a higher amount (fivefold) of enzyme is required for this purpose. The lipase from *P. fluorescens* catalyzes the acylation of nucleosides, using anhydrides in dimethylformamide (DMF) or dimethyl sulfoxide (DMSO), but poor regioselectivity is observed [23–25]. Following an opposite hydrolytic strategy, regioselective deacylations can be achieved either with *P. fluorescens* lipase (3'-position) or with subtilisin and/or an alkaline protease from *Bacillus subtilis* (5'-position) [23,24], as shown in Figure 14.6.

The highly regiospecific acylation of the OH group in C-2' remains unsolved. Only lipase from *Mucor javanicus* shows a moderated regioselectivity for this position (42% yield), using *n*-octanoic anhydride as the acyl donor [12]. Another plausible alternative is the deprotection of peracetylated nucleosides using two enzymatic steps [13]. Recently, wheat germ lipase (WGL) has been used to deacylate in one step C-5' and C-3' positions, but only moderated yields (26 to 29%) are reported [13].

Finally, Wang et al. [26] have described a highly diastereoselective thermophilic lipase ESL-001-02 that afforded in phosphate buffer at 60°C α -L-talofuranosyluronamide uracil (34) with 100% diastereomeric excess (de) from 5'-O-acetyl- β -D-allofuranosyl-uronamide uracil (33) (Figure 14.7).

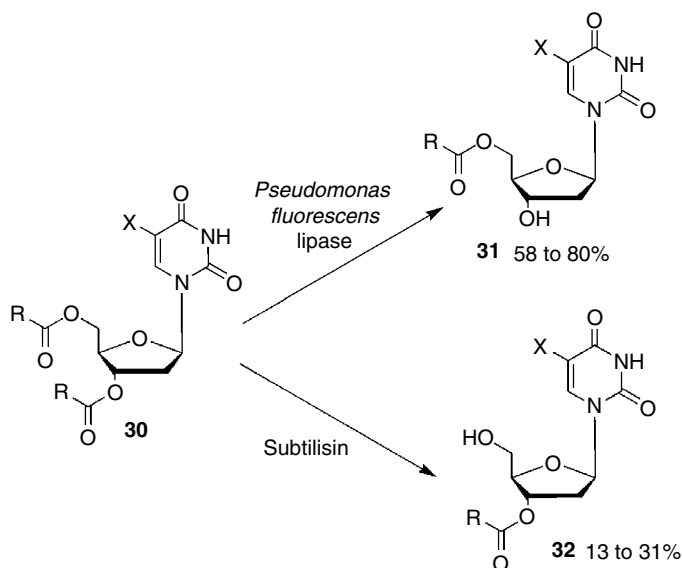


FIGURE 14.6 Regioselective deacylation of peracylated nucleosides using different enzymes. (From García, J., Fernández, S., Ferrero, M., et al., *Tetrahedron Lett.*, 45(8), 1709–1712, 2004.)

14.2.3 ENZYMIC OXIDATION OF CH₂OH IN NUCLEOSIDE ANALOGS

The enzymatic oxidation of these polyfunctional and labile compounds is more advantageous than the chemical process, because it is environmentally cleaner, and the avoiding of protection–deprotection steps of other groups makes it more attractive from an economical point of view. In fact, the preparation of carboxylic nucleoside derivatives in C-5' is a powerful tool for obtaining a new family of anti-inflammatory drugs [3,12]. In this sense, nucleoside oxidase from *Stenotrophomonas maltophilia* FERM BP-2252 has been used for this purpose [12,27]. This enzyme shows high tolerance for different functionalities in the base, especially in C-2 position, as shown in Figure 14.8. Furthermore, the N-1 can be modified either to N-oxide or N-methylated groups.

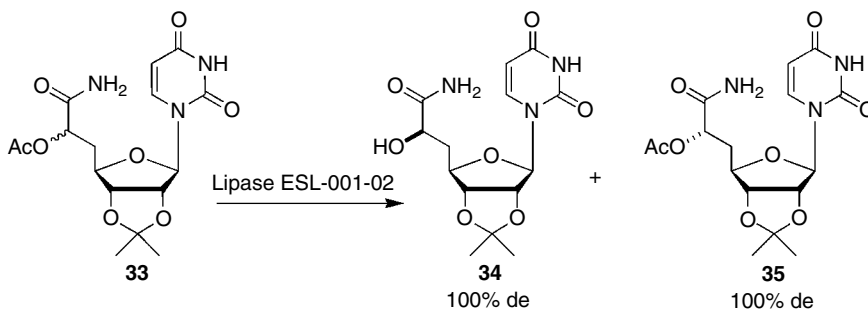


FIGURE 14.7 Selective hydrolysis of 5'-O-acetyl-β-D-allofuranosyluronamide uracil by lipase ESL-001-02. (From Wang, J-Q., Archer, C., Li, J., et al., *J. Org. Chem.*, 63(14), 4850–4853, 1998.)

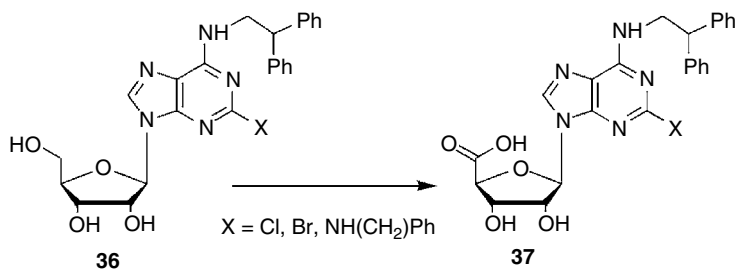


FIGURE 14.8 Selective oxidation of C-5 OH by oxidase from *Stenotrophomonas maltophilia*. (From Zhang, F.J. and Sih, C.J., *Tetrahedron Lett.*, 36(51), 9289–9292, 1995.)

14.2.4 ENZYMES THAT CATALYZE THE SYNTHESIS OF NUCLEOSIDES BY TRANSGLYCOSYLATION

Nucleoside analogs can be prepared by base interchange using two different kinds of intracellular enzymes: nucleoside phosphorylases (NP) and *N*-2'-deoxyribosyl transferases.

NPs catalyze the reversible phosphorylisis of nucleosides and the transferase reaction involving purine or pyrimidine bases. Purine (E.C. 2.4.2.2) and pyrimidine (E.C. 2.4.2.1) nucleoside phosphorylases have been isolated from a large number of bacteria [28]. These enzymes display fairly broad substrate specificity. Contrarily, *N*-2'-deoxyribosyl transferases (E.C. 2.4.2.6.) specifically catalyze the exchange of the base from a 2'-deoxyribosyl nucleoside with a free purine or pyrimidine [29], and especially those from *Lactobacilli* are well documented [30–33]. Both types of enzymes display a high regio- (*N*-1 glycosylation in pyrimidine and *N*-9 in purine) and stereoselectivity (β -anomers are exclusively formed). The formal synthetic process could be pictured as shown in Figure 14.9.

In order to find new active strains that are able to catalyze this process, a taxonomic screening of 147 microorganisms was performed in our laboratories, using strains belonging to different microbial groups: Bacillaceae, Enterobacteraceae, Lactobacillaceae, Photobacteraceae, Pseudomonaceae, Psychrobacter, and Vibrionaceae. Two reaction tests (Figure 14.10) were used in the screening: reaction test I was the synthesis of adenosine from uridine, and reaction test II was the synthesis of 2'-deoxyadenosine from 2'-deoxyuridine.

The screening resulted in 41 microorganisms (28%) active in reaction test I, while 53 (36%) microorganisms gave positive results in reaction test II, and 35 strains (24%) were positive in both reactions. Only six microorganisms were specific for the synthesis of ribonucleosides.

The most interesting microorganisms were selected according to four main criteria:

1. Low production of hypoxanthine (low adenosine deaminase activity)
2. Nonpathogenic and easy to cultivate in standard culture media

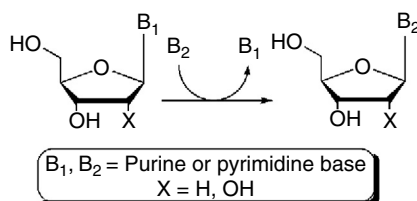


FIGURE 14.9 Synthetic activity of nucleoside phosphorylases (NPs).

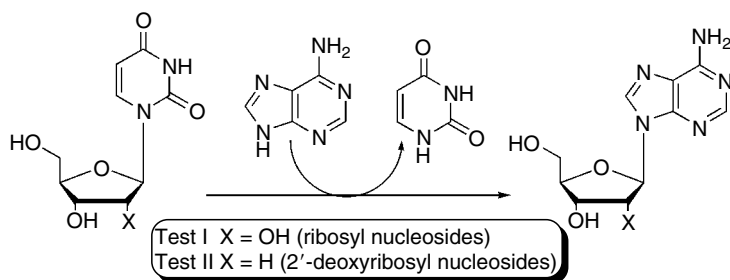


FIGURE 14.10 Test reactions used in the screening.

3. Ability to catalyze the reactions at low temperature
4. High productivity in adenosine or 2'-deoxyadenosine

Using these criteria, the microorganisms shown in Table 14.1 were selected as the most interesting strains for the synthesis of ribonucleosides, while those in Table 14.2 show the best strains for the synthesis of 2'-deoxyribosyl nucleosides. The assays were performed in duplicate experiments. In all cases, the reproducibility of the results was good, as we show in the case of *Xanthomonas translucens* (57°C, Table 14.1), and for *B. psychrosaccharolyticus* (57°C) and *Psychrobacter immobilis* (70°C) in Table 14.2. These two strains, and both *Photobacterium*, are described in this chapter for the first time as nucleoside phosphorylase producers.

TABLE 14.1

Microorganisms—Active in Reaction Test I and/or in Reaction Test II—Selected after the Screening Assays (Adenine = Uridine = 2'Deoxyuridine = 5 mM, Reaction Volume = 20 mL of 30 mM Phosphate Buffer (pH = 7), Time = 1 h)

Strain	T (°C)	Productivity [mM/(h×10 ⁶ cells)]×10 ⁵	Productivity (mM/(h×10 ⁶ cells) ×10 ⁵
		Reaction Test I	Reaction Test II
<i>Aeromonas salmonicida</i> ssp. <i>achromogenes</i> CECT 895	57	10.0	0
<i>Bacillus cereus</i> CECT 131	57	3.3	0
	70	1.72	6.17
	57	3.3	0
<i>B. subtilis</i> CECT 4524	70	1.1	4.5
<i>B. subtilis</i> ssp. <i>niger</i> CECT 4071	57	6.7	0
<i>Enterobacter amnigenus</i> CECT 4078	70	9.0	1.25
<i>E. sakazakii</i> CECT 858	70	9.0	2.25
<i>E. gergoviae</i> CECT 857	70	18.0	12.25
	70	15.5	20.5
<i>Erwinia amylovora</i> CECT 222	70	20.8	0
<i>Photobacterium leiognathi</i> CECT 4191	57	12.1	2.2 (70°C)
<i>P. phosphoreum</i> CECT 4192	57	11.6	0
<i>Serratia marcescens</i> CECT 977	57	7.6	4.01
	70	6.6	0
<i>S. rubidea</i> CECT 868	70	19.7	0
<i>Xanthomonas translucens</i> CECT 4643	57	15.5	12.8
	57	18.8	13.4

TABLE 14.2

Productivity Obtained in the Synthesis of 2'-Deoxyadenosine from 2'-Deoxyuridine. Specific Strains in the Synthesis of 2'-Deoxyribosyl Nucleosides (Adenine = 2'-Deoxyuridine = 5 mM, Reaction Time = 1 h; Reaction Volume = 50 mL)

Strain	<i>T</i> (°C)	Yield (%)	[Cells] (10 ⁶ cells/mL) ^a	Productivity [mM/(h × 10 ⁶ cells)] × 10 ⁵
<i>Bacillus coagulans</i> CECT 12	57	14	514	6.8
	70	24	1204	2.9
	70	13	1404	2.3
<i>B. psychrosaccharolyticus</i> CECT 4074	57	71	554	32
	57	56	590	27
<i>Enterobacter aerogenes</i> CECT 684 ^b	70	42	686	15
<i>Lactobacillus alimentarius</i> CECT 570 ^b	57	19	1677	2.8
	70	6.5	750	2.2
<i>L. pampilonensis</i> CECT 4219 ^b	57	42	2068	5.0
	70	7.7	1623	1.2
	70	8.8	2223	1.0
<i>Psychrobacter immobilis</i> CECT 4492	57	66	690	23
	57	53	713	18

^aCell cultured at 28°C.

^bHypoxanthine producer (yield > 10% referred to adenine).

Regarding the synthesis of ribonucleosides, some strains yielded adenosine without detectable production of hypoxanthine at $57 \pm 2^\circ\text{C}$: *B. cereus*, *B. subtilis*, *B. atrophaeus*, *Serratia marcescens*, and *X. translucens* (Table 14.1). This temperature is lower than that usually described for other strains in the synthesis of this nucleoside, which is normally carried out at temperatures higher than 60°C in order to reduce the production of hypoxanthine [33,34]. The productivity values for 2'-deoxyadenosine (Table 14.1) are generally lower than those obtained for adenosine in the case of nonspecific strains (Table 14.1). Nevertheless, *B. cereus* (70°C), *B. subtilis* (70°C), *Enterobacter gergoviae*, *S. marcescens*, and *X. translucens* showed good productivity in both reactions. It has been previously reported [35–37] that *B. subtilis*, *S. marcescens*, several *Enterobacter* strains, and *X. campestris* AJ 2797 are nucleoside phosphorylase producers.

From the results presented in Table 14.2 we can deduce that the most interesting strains (considering the productivity obtained) are *B. coagulans*, *B. psychrosaccharolyticus*, and *P. immobilis*. These strains displayed the best productivity values and the lowest hypoxanthine levels in reactions at 57°C . It must be considered that the presence of 2'-deoxyribosyl transferases in *Lactobacillus* spp. [30,31] is well documented, but that is not the case for psychrotrophic strains [38].

14.2.5 Characterization of the Enzymes Involved in the Biotransformation of N-2'-Deoxyribosyl Nucleosides

As indicated in Table 14.2, six strains were selected as active and specific for modification of 2'-deoxyribosyl nucleosides. Two different enzymes can catalyze the synthesis of 2'-deoxyribonucleosides by means of base interchange. The mechanism proposed for 2'-deoxyribosyl transferases involves a covalent catalysis, by analogy with the glycoside hydrolases that use

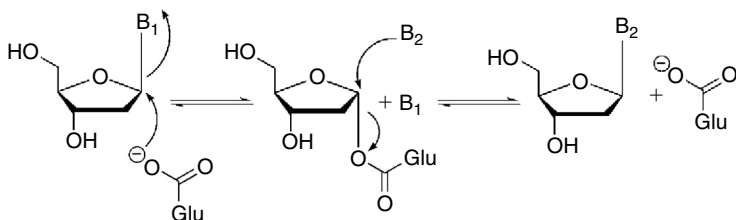


FIGURE 14.11 Mechanism proposed for 2'-deoxyribosyl transferases.

either a glutamyl or an aspartyl residue for catalytic activity. Indeed, several researchers [29,39,40] have demonstrated the presence of a glutamyl residue involved in the rate-controlling step in several *N*-2'-deoxyribosyl transferases, as depicted in Figure 14.11.

Another possibility involves two enzymes: thymidine nucleoside phosphorylase (TNP) and purine nucleoside phosphorylase [41,42]. TNP (E.C. 2.4.2.4) is specific for thymidine and the reaction takes place in two steps, through a 2'-deoxyribose-1-phosphate intermediate, as shown in Figure 14.12. TNP recognizes thymidine better than 2'-deoxyuridine [39,40].

The difference between both mechanisms is the presence of the ribose 1- α -phosphate intermediate in the case of TNP but not in the case of *N*-2'-deoxyribosyl transferases. Therefore, two parallel experiments were performed at the same time and with the same microbial culture. One reaction was performed using 30 mM NaH_2PO_4 / Na_2HPO_4 buffer (pH = 7) and the other using 25 mM Tris/HCl buffer (pH = 7). When the microorganisms expressed a TNP, the yield and productivity of the reaction were higher in phosphate buffer than in the presence of Tris/HCl buffer. Contrarily, if the microorganisms expressed a nucleoside 2'-deoxyribosyl transferase, the yield were similar in both reactions.

From the results in Table 14.3 we can deduce that *B. subtilis* (nonspecific strain, Table 14.1) cannot give the test II in the presence of Tris buffer. *E. aerogenes* clearly express a TNP because the reaction does not take place in Tris/HCl buffer. On the contrary, *B. psychrosaccharolyticus*, *B. coagulans*, *P. immobilis*, and *Lactobacillus pampilonensis* gave similar yields in Tris/HCl buffer as in phosphate buffer. This confirms the presence of nucleoside 2'-deoxyribosyl transferases in these strains.

14.2.6 IMMOBILIZATION OF WHOLE CELLS

Nucleoside phosphorylases are extracellular and multimeric enzymes [43,44]. A synthesis with these enzymes at semi-industrial scale would demand a co-immobilization step after the overexpression of the enzymes in genetically modified microorganisms and disruption of cells by consecutive passage of biomass in a high-pressure homogenizer [45–47]. Therefore, the immobilization–stabilization of these enzymes is a rather complex, but not impossible, process. In fact, nucleoside phosphorylases from *B. subtilis* have been expressed in *Escherichia*

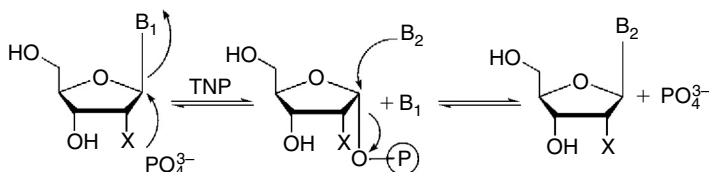


FIGURE 14.12 Mechanism involving thymidine and purine nucleoside phosphorylases.

TABLE 14.3

Productivity Obtained in the Synthesis of 2'-Deoxyadenosine from 2'-Deoxyuridine + Adenine in Tris/HCl or in Phosphate Buffer ($T = 57^{\circ}\text{C}$, Reaction Volume = 4 mL, 2'-Deoxyuridine = Adenine = 5 mM, Reaction Time = 20 min)

Strain	Buffer	Yield ^a (%)	Productivity [mM/(h $\times 10^6$ cells)] $\times 10^5$
<i>Bacillus coagulans</i>	Tris/HCl	15 (8)	3.7
CECT 12	Phosphate	14 (9)	3.8
<i>B. subtilis</i>	Tris/HCl	0	0
CECT 4524	Phosphate	35 (7)	31.5
<i>B. psychrosaccharolyticus</i>	Tris/HCl	48	31
CECT 4074	Phosphate	62	35
<i>Enterobacter aerogenes</i> ^b	Tris/HCl	0	0
CECT 4074	Phosphate	14 (6)	17
<i>Lactobacillus pampilonensis</i>	Tris/HCl	26 (8)	3.1
CECT 4219	Phosphate	23 (11)	2.7
<i>Psychrobacter immobilis</i>	Tris/HCl	27	13
CECT 4492	Phosphate	24	10

^aYield in 2'-deoxyadenosine and (yield in hypoxanthine).

^bReaction time when it is not 1 h.

coli, purified, and immobilized in sepabeads with polyethyleneamine, yielding an enzymatic derivative that is very active at pH = 10 and 45°C [46,47].

A cheap alternative is the immobilization of the whole cells [48,49]. With these biocatalysts, efficient syntheses of some purine nucleosides have been reported using *Erwinia herbicola* [34], *E. coli* BL21 [35,50], *B. stearothermophilus* [28], and *B. cereus* T. [51]. In order to scale up the synthetic process using whole cells, two main conditions are necessary:

1. Absence of secondary reactions such as deamination of adenine in adenosine ring
2. Possibility of reusing the biocatalyst [34,52,53]

Thus, the most widely used technique for cell immobilization is the cell entrapment [54], in which the living cells are enclosed in a polymeric porous matrix that allows the diffusion of substrates and products. The main advantages of this methodology are the high operational stability, the ease of cell handling and separation, and finally the feasibility of the scaling up [55–61].

Therefore, the best microorganisms as deduced by their performance shown in Table 14.1 and Table 14.2 were immobilized in different supports, in order to select the best biocatalyst according to the maximum yield reached and the maximum number of reuse cycles. The immobilized derivatives in agars (with different methoxylation degree) or in agaroses provided the best results. Different shapes of the biocatalysts—small beads, microbeads, or small parallelepipeds—were prepared in our laboratories, while the immobilization in microbeads was rejected because the biocatalysts were not interesting from the reuse and scale-up aspects, as we show in Figure 14.13 compared to Figure 14.14 (immobilization in small plates).

The qualitative behavior of other microorganisms immobilized in these matrixes was similar. This finding shows that all thermogels (obtained at their optimum percentage and with optimum shaking speed) do not exert dramatic restrictions on the diffusion of either reagents or products for obtaining an optimum yield in adenosine. In Figure 14.15 we observe

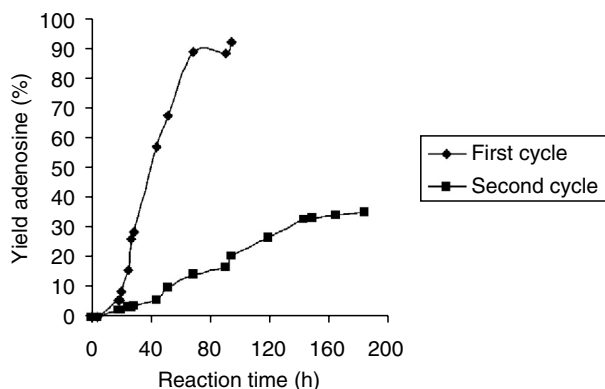


FIGURE 14.13 Synthesis of adenosine from uridine/adenine (30/10) mM using immobilized *Serratia marcescens* CECT 977 whole cells. Microbeads of highly methoxylated agar A28/03. Temperature = 57°C. Stirring speed = 250 r.p.m. Reaction time = 120 h. 40 g of wet biocatalyst.

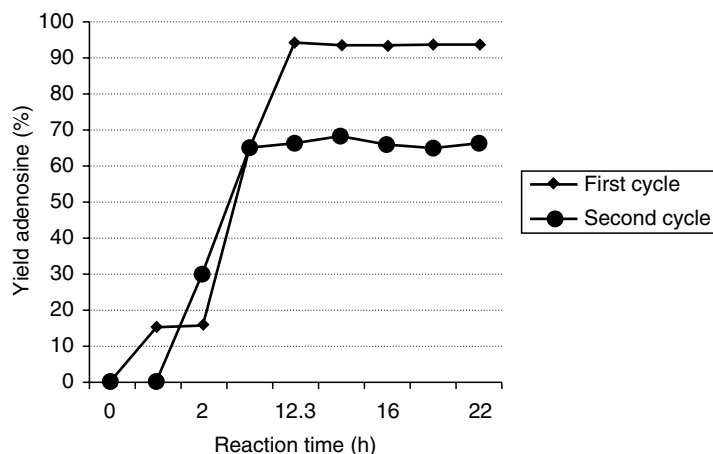


FIGURE 14.14 Synthesis of adenosine from uridine/adenine (30/10) mM using immobilized *Serratia marcescens* CECT 977 whole cells. Microplates (1 cm × 1 cm × 0.2 cm) of highly methoxylated agar A28/03. Temperature = 57°C. Stirring speed = 250 r.p.m. Reaction time = 120 h. 40 g of wet biocatalyst.

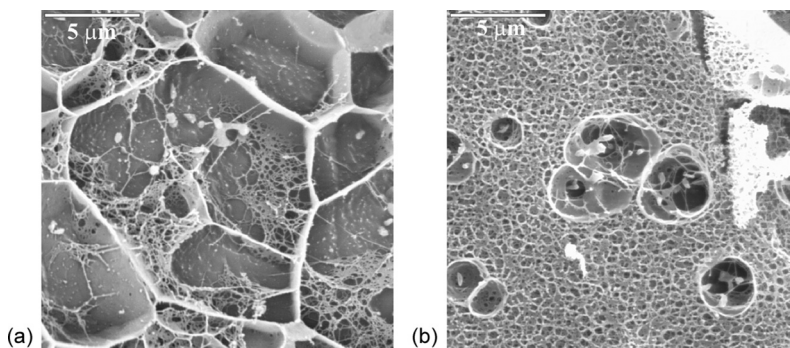


FIGURE 14.15 Electronic micrographs of immobilized whole cells of *Serratia marcescens* CECT 977 in (a) agar A28/03 or in (b) agarose.

that the bacteria are immobilized inside small cavities where only a few cells are entrapped, linked to the matrix through small linkers that probably could be mucopolysaccharides secreted by the cell. These microphotographs justify that in the washing of the biocatalyst particles for reusing, no free bacteria were observed in the reaction medium with a conventional microscope.

The stability of the biocatalysts in standard storage conditions (4°C) was also tested. After 60 to 75 days all the immobilized biocatalysts retained the same activity in the synthesis of adenosine starting from uridine and adenine [38].

In Table 14.4 we compare the catalytic activity of several strains immobilized in agar or agarose microplates using resting cell conditions. We can observe that *E. amnigenus* and *E. gergoviae* gave similar yields in all cases, while *S. marcenses* showed a moderated diminution in the yield in adenosine after immobilization. So, these biocatalysts can be used for a further scaling up.

In the case of the synthesis of 2'-deoxyribonucleosides, the immobilized whole cells in agar (A28/03) were as active in agar as in agarose, as we show for a nonspecific strain such as *E. gergoviae* (Table 14.5).

Even though better yield was obtained using the immobilized biocatalysts compared to free resting cells, the productivity was lower (Table 14.5), due to the longer reaction times demanded for the immobilized cells. Finally the best biocatalyst—*E. gergoviae* immobilized in small plates of highly methoxylated agar—was reused several times, as shown in Figure 14.16. This biocatalyst could be reused at least seven times without diminution of the yield. The immobilized biocatalysts in agarose gave good yield in the first cycle (Table 14.5), but only 60% yield was achieved in the second cycle. The yields obtained with immobilized *E. gergoviae* were similar to those described by Yokozeki and Tsuji [53], using free cells of *E. aerogenes* AJ-11125, or better than those described by Prasad et al. [62].

14.2.7 SYNTHESIS OF DIFFERENT NUCLEOSIDE ANALOGS

The syntheses of several nucleosids analogs have been described in the literature using whole cells as biocatalysts [36,49,63–66] (Table 14.6).

Kulikowska et al. [64] showed that 7-methylguanosine and 7-methylinosine are substrates of purine nucleoside phosphorylases. Taking into account this finding, Hennen and Wong

TABLE 14.4
Catalytic Activity of the Most Interesting Strains in the Synthesis of Adenosine from Uridine. Uridine/Adenine (30 mL/10 mM). Resting Cells and Immobilized in Agar A28/03 and in Agarose D5 ($T = 70^{\circ}\text{C}$, Reaction Time = 30 min (Resting Cells) or 1 h (Immobilized Cells), Reaction Volume = 15 ML, Wet Catalyst Weight = 20 g)

Microorganisms	Adenosine (%) Resting Cells	Adenosine (%) Immobilized Agar	Adenosine (%) Immobilized Agarose
<i>Enterobacter amnigenus</i> CECT 4078	98.5	88	94
<i>E. gergoviae</i> CECT 857	100	97	95
<i>E. sakazakii</i> CECT 858	91	5.2	48
<i>Serratia rubidea</i> CECT 868	90	11	7.5
<i>S. marcenses</i> CECT 977	100	70	70

TABLE 14.5

Productivity Values in the Synthesis of 2'-Deoxyadenosine from 2'-Desoxyuridine/Adenine (30 mL/10 mM) Using *Enterobacter gergoviae* in Resting Cells Condition (1 h) or Immobilized in Agar A28/03 (3 h) or in Agarose D5 (3 h) (Reaction Time = 30 min (Resting Cells) and 2 h (Immobilized Biocatalysts), 40 g of Wet Biocatalyst, $T = 70^{\circ}\text{C}$)

Catalyst	2'-Desoxyadenosine (%)	Cell Concentration (10^6 cell/ML)	Catalyst Load (10^6 cell/g cat)	Productivity [mM/(h $\times 10^6$ cells)] $\times 10^5$
Resting cells	42	1319	—	84
Immobilized agar A28/03	90	—	1319	23
Immobilized agarose	85	—	1110	25.5
Resting cells	43	1319	—	86

[65] proposed the use of 7-methylguanosinium or 7-methylinosinium as water-soluble glycosyl donor to obtain analogs of nucleosides in an irreversible and quantitative process, caused by the low solubility of guanine and inosine at $\text{pH} = 7.0$ to 7.4 (Table 14.7).

Shirae et al. [67] described the use of different *E. coli* strains (resting cells) for the preparation of different nucleoside analogs, such as 2',3'-dideoxyadenosine (**39**) or 2',3'-dideoxyinosine (**40**) (Figure 14.17). After a taxonomic screening of 436 microorganisms belonging to 39 genera, they selected one strain (optimal pH was ~ 6.5 , and optimal temperature was 50°C). These authors indicated that 2',3'-dideoxypyrimidine nucleosides (**38** and **41**) could also be obtained from 2',3'-dideoxyadenosine (**39**), but with moderated yields, as shown in Figure 14.17, so this result indicates the great versatility of the strain.

Recombinant *E. coli* BL21 strain was used by Rogert et al. [50] to prepare some *ara*-nucleosides (**43** and **44**) from *ara*-uridine (**42**) using resting cells at 60°C and $\text{pH} = 7.0$ (Figure 14.17). On the other hand, Murakami et al. [68] described the use of *E. coli* JA-300

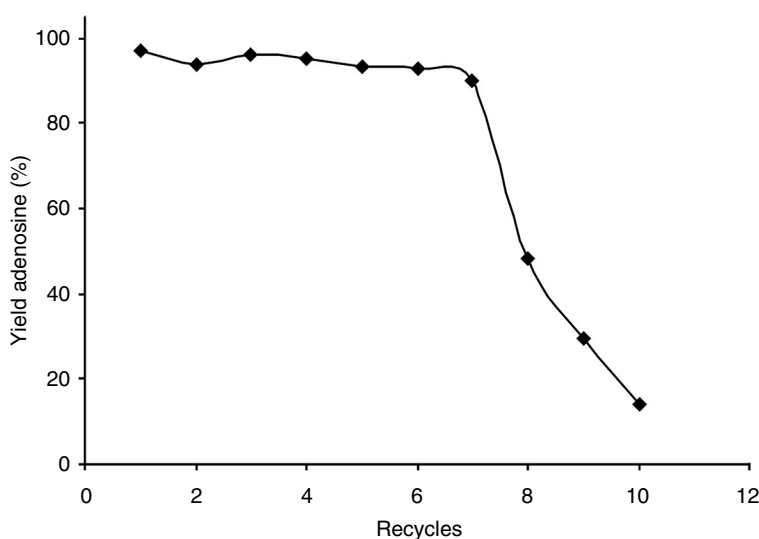
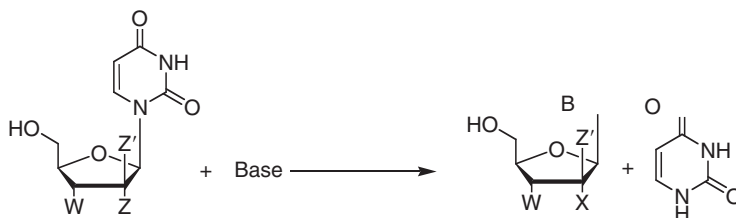


FIGURE 14.16 Recycling of immobilized biocatalysts: whole cells of *Enterobacter gergoviae* CECT 857 immobilized in small plates of agar A28/03, $T = 57^{\circ}\text{C}$.

TABLE 14.6

Synthesis of Some Nucleoside Analogs by Base Interchange (Enzymes: Pyrimidine + Purine Nucleoside Phosphorylase)



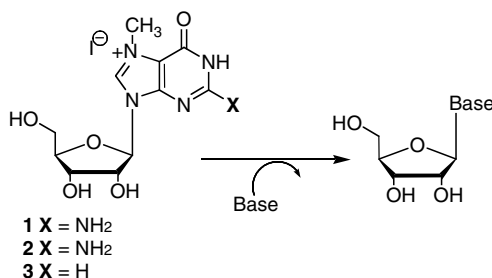
Pyridine Nucleoside	W	Z	Z'	Microorganism	Base	Yield in Nucleoside (%)	Time (h)	Ref.
Uridine	OH	OH	H	<i>E. gergoviae</i> CECT 857	6-Mercapto-purine	56	3	33
2'-Deoxyuridine	OH	H	H		6-Mercapto-purine	18	3	33
Uridine	OH	OH	H		3-Carboxy-amido 1,2,4-triazol	45	3	33
Uridine	OH	H	H		Purine	80	3	33
Ara-uridine	OH	H	OH	<i>E. coli</i> BMT-ID/1A	Adenine	83	133	55
Uridine	OH	OH	H	<i>E. coli</i> free enzymes	4-Chloro-imidazo [4,5-c] pyrimidine	57	—	59

whole cells (50°C and pH = 6.5) in the synthesis of 6-halo-2',3'-dideoxypurine nucleosides (**45** and **46**), used as anti-HIV drugs, starting from 2',3'-dideoxyuridine (**38**) (Figure 14.17).

Finally, recombinant *E. coli* BM-11 cells have been used by Zinchenko et al. [69] to obtain 9-(β-D-arabinofuranosyl)guanine (**47**, Figure 14.18), active against viruses of herpes type and also for inhibiting T-lymphocyte proliferation. The synthesis was performed from 1-(β-D-arabinofuranosyl)uracil (**42**) and three different guanine donors: guanine (**48**), guanosine (**4a**) and 2'-deoxyguanosine (**49**), as shown in Figure 14.18.

TABLE 14.7

Synthesis of Some Nucleoside Analogs Using 7-Methyl Guanidinium or 7-Methylinosinium Salts



Initial Compound ^a	Base ^b	Yield in Nucleoside	Reaction Time (days)
1	adenine	100	4
2	Adenine	100	4
3	Adenine	100	2
2	3-Diazaadenine	70	2
1	3-Diazaadenine	50	2
2	1,2,4-Triazol-3-carboxiamide	60	2
3	1,2,4-Triazol-3-carboxiamide	57	2

^a100 mol.^b25 mol.

Source: Hennen, W.J. and Wong, Ch-H., *J. Org. Chem.*, 54(19), 4692–4695, 1989.

The synthesis starting from guanine nucleosides leads to higher yields than using free base. The phenomenon is related to the poor solubility of guanine in the reaction conditions (~0.042 g/L) which leads to a low base concentration and thus the unfeasibility of an effective transglycosidation mediated by the cells. Contrarily, both nucleosides are used by the cells to render a high yield in *ara*-guanosine. As can be seen, the cross-linking with glutaraldehyde of whole cells increases both the stability of biocatalysts and the obtained yield.

Some other researchers have described the synthesis of nucleoside analogs using phosphorylases in combination with other enzymes. Thus, Pal and Nair [28] described how the addition of xanthineoxidase (xodase) to a synthesis of nucleosides catalyzed by whole cells of *B. stearothermophilus* ATCC 12980 leads to higher yields in the synthesis of thymidine (**51**) starting from 2'-deoxyinosine (**50**) than those described in the absence of xodase (Figure 14.19). This fact is explained because xodase shifts the equilibrium of the reversible transglycosylation

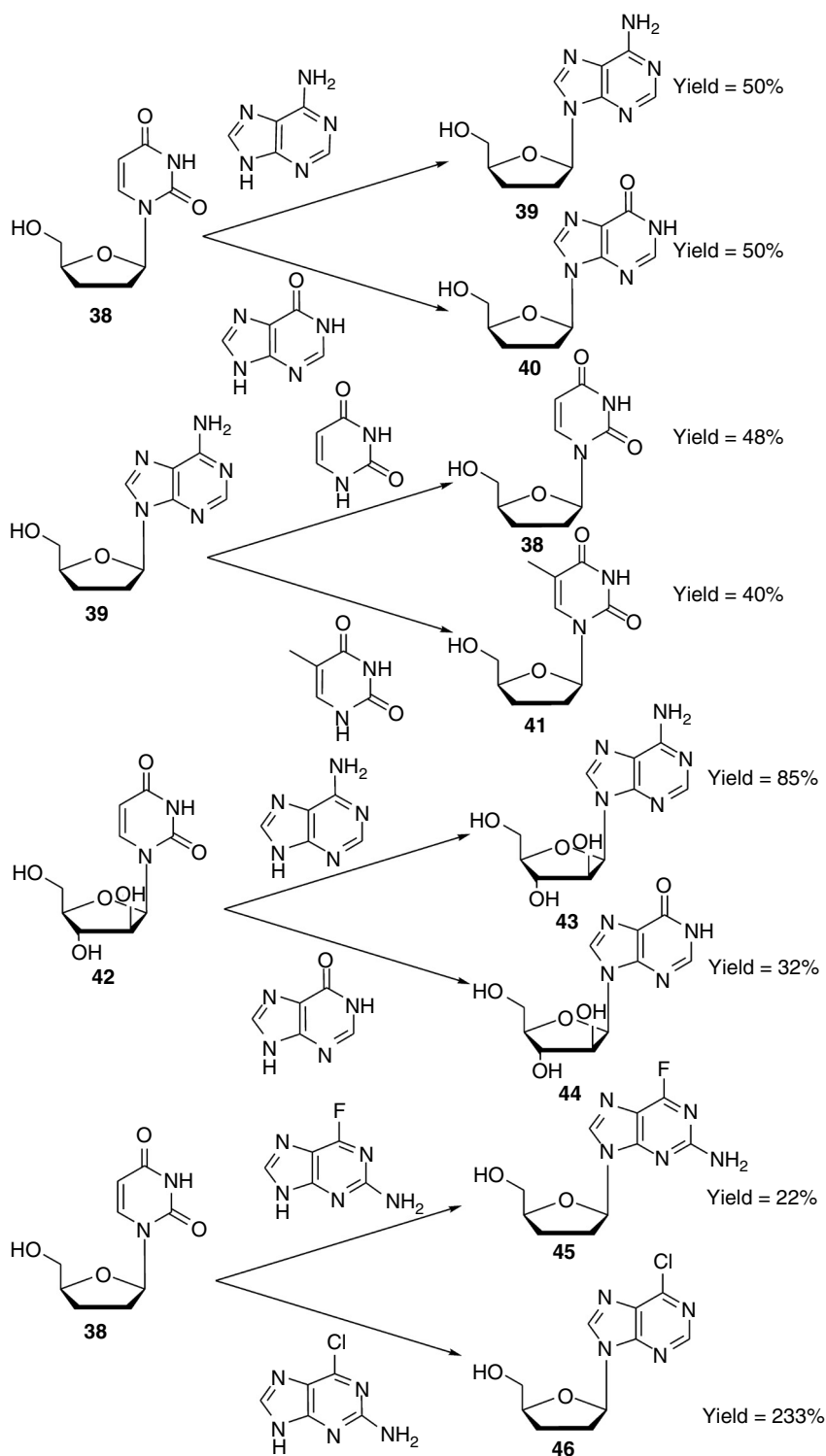


FIGURE 14.17 Synthesis of different nucleoside analogs using some *Escherichia coli* strains.

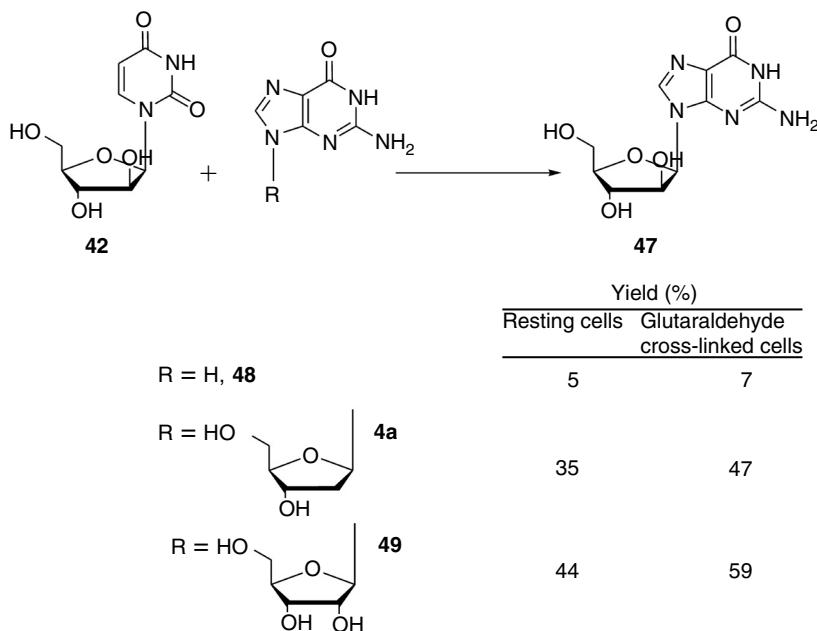


FIGURE 14.18 Synthesis of 9-(β-D-arabinofuranosyl) guanine using free cells and cross-linked cells of *Escherichia coli* BM-11. (From Zinchenko, A.I., Barai, V.N., Bokut, S.B., et al., *Appl. Microbiol. Biotechnol.*, 32(6), 658–661, 1990.)

reaction toward completion by the formation of uric acid (**52**, Figure 14.19), which is not recognized by phosphorylase.

Finally, Yokozeki and Tsuji [53] described the synthesis of 2'-deoxyguanosine (**4a**, Figure 14.20) with 100% yield at 25°C, using *E. gergoviae* AJ-11125 whole cells. This strain has a very active adenosine deaminase, so that the synthesis of **4a** could be described using

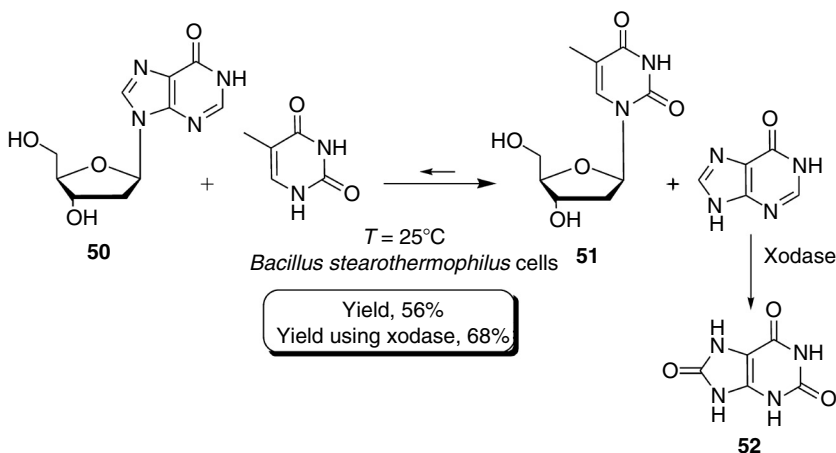


FIGURE 14.19 Synthesis of nucleoside analogs using phosphorylases in combination with xanthineoxidase. (From Pal, S. and Nair, V., *Biocatal. Biotransfor.*, 15(2), 147–158, 1997.)

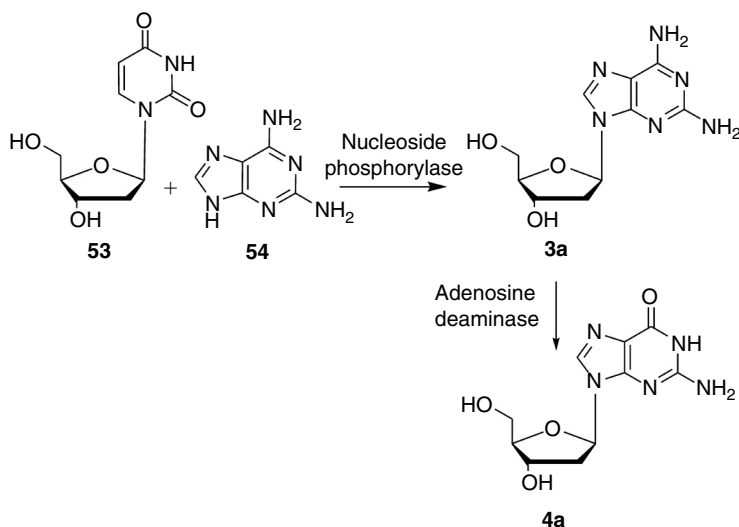


FIGURE 14.20 Synthesis of nucleoside analogs using phosphorylases in combination with adenosine deaminase (ADA). (From Yokozeki, K. and Tsuji, T., *J. Mol. Catal. B Enzym.*, 10(1–3), 207–213, 2000.)

2,6-diaminopurine (**54**) and 2'-deoxyuracil (**53**) as base acceptor, through **3a** intermediate (Figure 14.20). This indirect methodology overcomes the direct synthesis of **4a** from guanine and **53** due to the low water solubility of the base in the medium.

14.3 CONCLUSIONS

The enzymatic synthesis of nucleoside analogs using either free enzymes or whole cells can be advantageous over chemical synthesis due to the reduction in the number of steps and the high regio- and stereoselectivity observed. Different enzymes can be used in the synthesis of these compounds in order to selectively modify either the base or the sugar functional groups. In addition, different expensive nucleoside analogs can be prepared starting from cheap nucleosides by base interchange using immobilized whole cells in processes that are easy to scale up. The main problem for this synthetic methodology is to design a “homogeneous reaction media” for both nucleosides (hydrophilic) and bases (hydrophobic). Therefore, the development of new solvents displaying low toxicity for cells or enzymes and possessing good solving properties is the main work in the future for scaling up these syntheses.

REFERENCES

1. Garg, R., Gupta, S.P., Gao, H., et al., Comparative quantitative structure–activity relationship studies on anti-HIV drugs, *Chem. Rev.*, 99(12), 3525–3601, 1999.
2. Buolamwimi, J.K., Nucleoside transport inhibitors: structure–activity relationships and potential therapeutic applications, *Curr. Med. Chem.*, 4(1), 35–66, 1997.
3. Mahmoudian, M., Rudd, B., Cox, B., et al., A versatile procedure for the generation of nucleoside 5'-carboxylic acids using nucleoside oxidase, *Tetrahedron*, 54(28), 8171–8182, 1998.
4. Fox, K.R., Targeting DNA with triplexes, *Curr. Med. Chem.*, 7(1), 17–37, 2000.
5. Rachakonda, S. and Cartee, L., Challenges in antimicrobial drug discovery and the potential of nucleoside antibiotics, *Curr. Med. Chem.*, 11(6), 775–793, 2004.

6. Pankiewicz, K.W., Fluorinated nucleoside, *Carbohydr. Res.*, 327(1–2), 87–105, 2000.
7. Gómez, J.A., Trujillo, M.A., Campos, J., et al., Synthesis of novel 5-fluorouracil derivatives with 1,4-oxaheteroepine moieties, *Tetrahedron*, 54(43), 13295–13312, 1998.
8. Priego, E.M., Balzarini, J., Karlsson, A., et al., Synthesis and evaluation of thymidine carboxamides against mitochondrial thymidine kinase (TK-2) and related enzymes, *Bioorg. Med. Chem.*, 12(19), 5079–5090, 2004.
9. Raic-Malic, S., Herold-Brudic, A., Nagl, A., et al., Novel pyrimidine and purine derivatives of L-ascorbic acid: synthesis and biological evaluation, *J. Med. Chem.*, 42(14), 2673–2678, 1999.
10. Ramesh, N., Klunder, A.J.H., and Zwanenburg, B., Enantioselective synthesis of 4-acetylaminocyclopent-2-en-1-ols from tricycle [5,2,1,0(2,6)] decenylenaminone: precursor for 5'-norcarbocyclic nucleosides and related antiviral compounds, *J. Org. Chem.*, 64(10), 3635–3641, 1999.
11. Harword, E.A., Hopkins, P.B., and Sigurdsson, S.Th., Chemical synthesis of cross-link lesions found in nitrous acid treated DNA: a general method for the preparation of N-2-substituted-2'-deoxyguanosine, *J. Org. Chem.*, 65(10), 2959–2964, 2000.
12. Ferrero, M. and Gotor, V., Chemoenzymatic transformations in nucleoside chemistry, *Monatsh. Chem.*, 131(6), 585–616, 2000.
13. Ferrero, M. and Gotor, V., Biocatalytic modifications of conventional nucleosides, carbocyclic nucleosides and C-nucleosides, *Chem. Rev.*, 100(12) 4319–4347, 2000.
14. Discroll, J.S., Siddiqui, M.A., Ford, H., Jr., et al., Lipophilic acid-stable, adenosine deaminase-activated anti-HIV prodrugs for central nervous system delivery, 3: 6-amino prodrugs of 2'- β -fluoro-2',3'-dideoxyinosine, *J. Med. Chem.*, 39(8), 1619–1625, 1996.
15. Santaniello, E., Ciuffreda, P., and Alessandrini, L., Synthesis of modified purine nucleosides and related compounds mediated by adenosine deaminase (ADA) and adenylylate deaminase, *Synthesis*, 4, 509–526, 2005.
16. Kakefuda, A., Shuto, S., Nagahata, T., et al., Nucleosides and nucleotides 132. Synthesis and biological evaluation of ring-expanded oxetanocin analogues: purine and pyrimidine analogues of 1,4-anhydrido-2-deoxy-D-arabitol and 1,4-anhydro-2-deoxy-3-hydroxymethyl-D-arabitol, *Tetrahedron*, 50(34), 10167–10182, 1994.
17. Hertel, L.W., Grossman, C.S., and Kroin, J.S., Method of preparing β -2',2'-difluoronucleosides, Eur. Pat. Appl. 0328345, 1989.
18. Vince, R. and Brownell, J., Resolution of racemic carbovir and selective inhibition of human immunodeficiency virus by the (–) enantiomer, *Biochem. Biophys. Res. Co.*, 168(3), 912–916, 1990.
19. Margolin, A.L., Borcherdig, D.R., Wolf-Kugel, et al., AMP deaminase as a novel practical catalyst in the synthesis of 6-oxopurine ribosides and their analogs, *J. Org. Chem.*, 59(24), 7214–7218, 1994.
20. Moris, F. and Gotor, V., A novel and convenient route to 3'-carbonates from unprotected 2'-deoxynucleosides through an enzymatic reaction, *J. Org. Chem.*, 57(8), 2490–2492, 1992.
21. Moris, F. and Gotor, V., A useful and versatile procedure for the acylation of nucleosides through an enzymatic reaction, *J. Org. Chem.*, 58(3), 653–660, 1993.
22. García, J., Fernández, S., Ferrero, M., et al., A mild, efficient and regioselective enzymatic procedure for 5'-O-benzoylation of 2'-deoxynucleosides, *Tetrahedron Lett.*, 45(8), 1709–1712, 2004.
23. Uemura, A., Nozaki, K., Yamashita, J.-L., et al., Lipase-catalyzed regioselective acylation of sugar moieties of nucleosides, *Tetrahedron Lett.*, 30(29), 3817–3818, 1989.
24. Fan H., Kitawa, M., Raku, T., et al., Enzymatic synthesis of vinyl uridine esters catalyzed by Bioprse, alkaline protease from *Bacillus subtilis*, *Biochem. Eng. J.*, 21(3), 279–283, 2004.
25. Uemura, A., Nozaki, K., Yamashita, J.-L., et al., Regioselective deprotection of 3',5'-O-acylated pyrimidine nucleosides by lipases and esterases, *Tetrahedron Lett.*, 30(29), 3819–3820, 1989.
26. Wang, J.-Q., Archer, C., Li, J., et al., Thermophilic esterases/lipases as an effective tool for the resolution of nucleoside diastereoisomers: convenient one-pot synthesis of α -L-taluronamide and β -D-alluronamide nucleosides, *J. Org. Chem.*, 63(14), 4850–4853, 1998.
27. Zhang, F.J. and Sih, C.J., Novel enzymatic cyclizations of pyrimidine nucleoside analogues: cyclic-GDP-ribose and cyclic-HDP ribose, *Tetrahedron Lett.*, 36(51), 9289–9292, 1995.
28. Pal, S. and Nair, V., Enzymatic synthesis of thymidine using bacterial whole cells and isolated purine nucleoside phosphorylase, *Biocatal. Biotransfor.*, 15(2), 147–158, 1997.

29. Short, S.A., Armstrong, S.R., Ealick, S.E., et al., Active site aminoacids that participate in the catalytic mechanism of nucleoside 2'-deoxyribosyltransferase, *J. Biol. Chem.*, 271(9), 4978–4987, 1996.
30. Cardinaud, R. and Holguin, J., Nucleoside deoxyriboxyltransferase-II from *Lactobacillus helveticus*. Substrate specificity studied: pyrimidine bases as acceptors, *Biochim. Biophys. Acta*, 568(2), 339–347, 1979.
31. Kaminski, P.A., Functional cloning, heterologous expression and purification of two different *N*-deoxyrybosyltransferases, *J. Biol. Chem.*, 277(17), 14400–14407, 2002.
32. Carson, D.A. and Wasson, D.B., Synthesis of 2',3'-dideoxynucleosides by enzymatic *trans*-glycosylation, *Biochem. Biophys. Res. Comm.*, 155(2), 829–834, 1988.
33. Huang, M.C., Montgomery, J.A., Thorpe, M.C., et al., Formation of 3'-(2'-deoxyribofuranosyl) and 9-(2'-deoxyribofuranosyl) nucleosides of 8-substituted purines by nucleoside deoxyriboxyltransferases, *Arch. Biochim. Biophys.*, 222(1), 133–144, 1983.
34. Utagawa, T., Enzymatic preparation of nucleoside antibiotics, *J. Mol. Catal. B Enzym.*, 6(3), 215–222, 1999.
35. Lewkowicz, E.S., Martínez, N., Rogert, M.C., et al., An improved microbial synthesis of purine nucleosides, *Biotechnol. Lett.*, 22(16), 1277–1280, 2000.
36. Hayashi, Y., Shiba, T., Katsuyama, A., et al., Method of manufacturing trifluorothymidin, Eur. Pat. Appl. 0331853, 1989.
37. Trelles, J.A., Fernández, M., Lewkowicz, E.S., et al., Purine nucleoside synthesis from uridine using immobilized *Enterobacter gergoviae* CECT 875 whole cells, *Tetrahedron Lett.*, 44(12), 2605–2609, 2003.
38. Sinisterra, J.V., Condezo, L.A., and Fernández-Lucas, J., Procedimiento para la síntesis de nucleósidos mediante la utilización de microorganismos psicrotrofos o psicrotolerantes, Spanish Pat. No. 200400817, PCT/ES/2005/00166, 2004.
39. Holguin, J., Cardinaud, R., and Salemnik, C.A., *trans-N*-deoxyriboxylase: substrate specificity studies. Purine bases as acceptors, *Eur. J. Biochem.*, 54(2), 515–520, 1975.
40. Danzin, C. and Cardinaud, R., Deoxyribosyltransfer catalysis with *trans-N*-deoxyriboxylase: kinetic study of purine (pyrimidine) to pyrimidine (purine) *trans-N*-deoxyriboxylase, *Eur. J. Biochem.*, 62(2), 365–372, 1976.
41. Walter, M.R., Cook, W.J., Cole, L.B., et al., Three-dimensional structure of thymidine phosphorylase from *E. coli* at 2.8 Å resolution, *J. Biol. Chem.*, 265(23), 14016–14022, 1990.
42. Zimmerman, M. and Seidenberg, J.I., Thymidine phosphorylase and nucleoside deoxyribosyltransferase in normal and malignant tissues, *J. Biol. Chem.*, 239(8), 2618–2621, 1964.
43. Pugmire, M.J. and Ealick, S.E., Structural analyses reveal two distinct families of nucleosidephosphorylases, *Biochem. J.*, 361, 1–25, 2002.
44. Erion, M.D., Stoecker, J.D., Guida, W.C., et al., Purine nucleoside phosphorylase 2: catalytic mechanism, *Biochemistry (USA)*, 36(39), 11735–11748, 1997.
45. Zuffi, G., Ghisotti, D., Oliva, I., et al., Immobilized biocatalysts for production of nucleosides and nucleoside analogues by enzymatic transglycosidation reactions, *Biocatal. Biotransfor.*, 22(1), 25–33, 2004.
46. Ubiali, D., Rocchiatti, S., Scaramozzin, F., et al., Synthesis of 2'-deoxynucleosides by transglycosilation with new immobilized and stabilized uridine phosphorylase and purine nucleoside phosphorylase, *Adv. Synth. Catal.*, 346(11), 1361–1366, 2004.
47. Rocchiatti, S., Ubiali, D., Terreni, M., et al., Immobilization and stabilization of recombinant multimeric uridine and purine nucleoside phosphorylases from *Bacillus subtilis*, *Biomacromolecules*, 5(6), 2195–2200, 2004.
48. Huang, J., Hooijmans, C.M., Briasco, C.A., et al., Effect of free-cell growth parameters on oxygen concentration profiles in gel-immobilized recombinant *E. coli*, *Appl. Microbiol. Biotechnol.*, 33(6), 619–623, 1990.
49. Trelles, J.A., Fernández-Lucas, J., Condezo L.A., et al., Nucleoside synthesis by immobilized bacterial whole cells, *J. Mol. Catal. B Enzym.*, 35(5–6), 219–227, 2004.
50. Rogert, M.C., Trelles, J.A., Porro, S., et al., Microbial synthesis of antiviral nucleosides using *Escherichia coli* BL21 as biocatalyst, *Biocatal. Biotransfor.*, 20(5), 347–351, 2002.

51. Gardner, R. and Kornberg, A., Biochemical studies of bacterial sporulation and germination, V: purine nucleoside phosphorylases of vegetative cells and spores of *Bacillus cereus*, *J. Biol. Chem.*, 242(10), 2383–2388, 1967.
52. Yokozeki, K., Shirae, H., Kobayashi, K., et al., Methods of producing 2',3'-dideoxyinosine, US Patent No. 4970148, 1990.
53. Yokozeki, K. and Tsuji, T., A novel enzymatic method for the production of purine-2'-deoxyribonucleosides, *J. Mol. Catal. B Enzym.*, 10(1–3), 207–213, 2000.
54. Mazid, M.A., Biocatalysis and immobilized/cell bioreactors, *Biotechnology*, 11(6), 690–695, 1993.
55. Bickerstaff G.F., Immobilization of enzymes and cells: some practical considerations, in *Immobilization of Enzymes and Cells*, Bickerstaff, G.F., Ed., Humana Press, Totowa, NJ, 1987, pp. 1–3.
56. Hulst, A.C., Tramper, J., Riet, K., et al., A new technique for production of immobilized biocatalyst in large quantities, *Biotechnol. Bioeng.*, 27(6), 870–876, 1995.
57. Quintana, M.G. and Dalton, H., Production of toluene *cis*-diol by immobilized *Pseudomonas putida* UV4 in barium alginate beads, *Enzyme Microb. Technol.*, 22(8), 713–720, 1998.
58. Khattar, J.I.S., Sarma, T.A., and Singh, D.P., Removal of chromium ions by agar immobilized cells of the cyanobacterium *Anacystis nidulans* in a continuous flow reactor, *Enzyme Microb. Technol.*, 25(7), 564–568, 1999.
59. Lozinsky, V.I. and Plieva, F.M., Poly(vinylalcohol) cryogels employ matrix for cell immobilization, 3: overview of recent research developments, *Enzyme Microb. Technol.*, 23(3–4), 227–242, 1998.
60. Sinisterra, J.V. and Dalton, H., Influence of the immobilization methodology in the stability and activity of *P. putida* UV4 immobilized whole cells, in *Immobilized cells: Basics & Applications*, Wijffels, R.H., Buitlear, R.M., Bucke, C., and Tramper, J., Eds., Elsevier Science, Amsterdam, 1996, pp. 416–423.
61. Carballeira, J.D., Valmaseda, M., Alvarez, E., et al., *Gongronella butleri*, *Schizosaccharomyces octosporu*, *Diplogelasinospora grovesii*: novel microorganisms useful for the stereoselective reduction of ketones, *Enzyme Microb. Technol.*, 34(6), 611–623, 2004.
62. Prasad, A.K., Trikha, S., and Parmar, V.S., Nucleoside synthesis mediated by glycosyl transferring enzymes, *Bioorg. Chem.* 27(2), 135–154, 1999.
63. Trelles, J.A., Betancor, L., Schoijer, A., et al., Immobilized *Escherichia coli* BL21 as a catalyst for the synthesis of adenine and hypoxanthine nucleosides, *Chem. Biodivers.*, 1(2), 280–288, 2004.
64. Kulikowska, E., Bzowska, A., Wierzechowski, J., et al., Properties of two unusual and fluorescent substrates of purine-nucleoside phosphorylase 7-methylguanosine and 7-methylinosine, *Biochim. Biophys. Acta.*, 874(3) 355–363, 1986.
65. Hennen, W.J. and Wong, Ch.-H., A new method for the enzymatic synthesis of nucleosides using purine nucleoside phosphorylase, *J. Org. Chem.*, 54(19), 4692–4695, 1989.
66. Krenitsky, T.A., Freeman, G.A., Shaver, S.R., et al., Imidazo [4,5-c]pyrimidines (3-deazapurines) by glycosyl transferring and their nucleosides as immunosuppressive and anti-inflammatory agents, *J. Med. Chem.*, 29(1), 138–143, 1986.
67. Shirae, H., Kobayashi K., Shiragami, H., et al., Production of 2',3'-dideoxyadenosine and 2',3'-dideoxyinosine from 2',3'-dideoxyuridine and the corresponding bases by resting cells of *Escherichia coli* AJ 2595, *Appl. Environ. Microbiol.*, 55(2), 419–424, 1989.
68. Murakami, K., Shirasaka, T., Yoshioka, H., et al., *Escherichia coli* mediated biosynthesis and *in vitro* anti-HIV activity of lipophilic 6-halo-2',3'-dideoxynucleosides, *J. Med. Chem.*, 34(5), 1606–1612, 1991.
69. Zinchenko, A.I., Barai, V.N., Bokut, S.B., et al., Synthesis of 9-(β -D-arabinofuranosyl) guanine using whole cells of *Escherichia coli*, *Appl. Microbiol. Biotechnol*, 32(6), 658–661, 1990.

15 Biocatalytic Reduction of Carboxylic Acids: Mechanism and Applications

*Padmesh Venkitasubramanian, Lacy Daniels,
and John P.N. Rosazza*

CONTENTS

15.1	Mechanism of Carboxylic Acid Reduction by CAR.....	427
15.2	CAR Contains a Phosphopantetheine Attachment Site.....	428
15.3	Evidence for CAR Phosphopantetheinylation	429
15.4	A Revised Mechanism for Holo-CAR Reduction of Carboxylic Acids.....	431
15.5	Applications of CAR	431
15.6	CAR is Versatile	432
15.7	CAR is Stereoselective	433
15.8	Synthesis of Value-Added Chemicals.....	436
15.8.1	Vanillin.....	436
15.8.2	Ferulic Acid	436
15.9	Conclusions.....	437
	Acknowledgments	438
	References	438

In nature, carboxylic acids are a ubiquitous class of organic compounds. They occur either as free acids like acetic, pyruvic, or citric or masked in the forms of esters, anhydrides, lactones amides, and lactams. Most free carboxylic acids occur as salts, leading to their chemical stability and relatively high solubilities in water. Carboxylic acids and their derivatives are often precursors for value-added chemicals for the pharmaceutical, agricultural, and food industries. Methods to harness this pool of valuable precursors have led to the development of several methods for chemical reductions and other manipulations of carboxylic acids.

Microorganisms and their enzymes are now widely used as classes of “biocatalytic reagents” in synthetic organic chemistry [1–12]. Biocatalysts intrinsically bind organic substrates, and catalyze highly specific and selective reactions under the mildest of reaction conditions. These selectivities and specificities are often realized because of highly rigid interactions occurring between enzyme active sites and substrate molecules. Microbial reductions of aromatic carboxylic acids, usually to their corresponding alcohols, have been observed with whole-cell biotransformations by a number of microorganisms including *Actinomyces* [13], *Clostridium thermoaceticum* [14], *Aspergillus niger* [15,16], *Corynespora melonis* [15], *Coriolus* [15], *Neurospora* [17], *Glomerella cingulata* [18,19], *Gloeosporium*

laeticolor [19], and *Nocardia* sp. [20,21]. In all reported cases, substrates contained an aromatic moiety, albeit not all directly attached to the carboxyl groups being reduced.

Gross and Zenk first showed that *Neurospora crassa* gave an aldehyde intermediate during carboxylic acid reductions [22,23]. The enzyme reducing aryl-carboxylic acids to aldehydes was purified and identified as a monomeric protein with an apparent molecular mass of 120,000 Da [22]. The reduction reaction was adenosine triphosphate (ATP)-, Mg^{2+} -, and NADPH-dependent [22]. The *N. crassa* aryl-aldehyde oxidoreductase (AAOR) (EC 1.2.1.30) or carboxylic acid reductase (CAR) initially catalyzed the condensation of carboxylic acid with ATP to yield an acyl-adenosine monophosphate (AMP) intermediate, which was subsequently reduced by NADPH to afford the aldehyde (Figure 15.1) [24]. Because the reaction is irreversible, AAOR (EC 1.2.1.30) is different from aldehyde oxidoreductases (EC 1.2.1.30), which oxidize aldehydes to carboxylic acids using NAD^+ [24,25].

Kato et al. showed that *Nocardia asteroides* JCM 3016 could reduce benzoic acid to benzyl alcohol [21]. Similar to the CAR from *Neurospora*, the reductase from this organism reduced benzoic acid to benzaldehyde using ATP, NADPH, and Mg^{2+} [26]. Reported K_m values for benzoic acid, NADPH, and ATP were extremely low at 260 nM, 6 nM, and 23 nM, respectively. This enzyme reduced a number of mono- and multisubstituted benzoic acids to corresponding aldehydes. This CAR also catalyzed the reduction of benzoyl-AMP. Although aryl-carboxylic acid reduction to aldehyde activities has been observed in many microorganisms [13–20], enzymes catalyzing this reaction have been purified only from *N. crassa* [22] and *N. asteroides* [26].

In our laboratory, a strain of *Nocardia* sp. NRRL 5646 efficiently reduced aryl-carboxylic acids to aldehydes and alcohols. The substrate specificity for reducing carboxylic acids with this organism appeared to be significantly different than that for the enzyme reported by Kato et al. [26]. The availability of relatively large amounts of pure CAR was necessary for elucidation of its properties and its catalytic mechanism. *Nocardia* sp. NRRL 5646 CAR was purified 196-fold to homogeneity by a combination of Mono-Q, Reactive Green 19 agarose affinity, and hydroxyapatite chromatographies. A key step was the binding and elution of CAR from Reactive Green 19 [27]. ATP, $NADP^+$, and NADPH were used to elute CAR from Reactive Green 19, suggesting that binding of the enzyme to this matrix was due to a CAR nucleotide binding site.

At 128,000 Da, the molecular mass of the *Nocardia* sp. NRRL 5646 CAR by SDS-PAGE and gel filtration chromatography was similar to that for CAR from *N. asteroides* [27] and *N. crassa* [22]. Apparent K_m values for benzoate, ATP, and NADPH for *Nocardia* sp. NRRL 5646 CAR were more than 1000-fold higher than those reported for the enzyme from *N. asteroides*

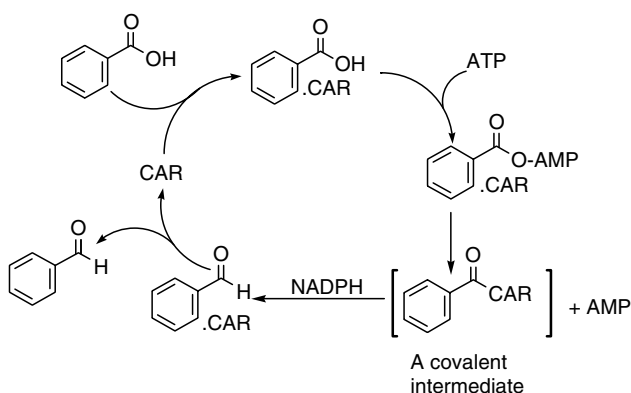


FIGURE 15.1 Enzyme catalyzed reduction of carboxylic acids.

[26]. The K_m of benzoate for *N. crassa* CAR was 63 μM and its activity was inhibited by 300 μM benzoate [23]. No inhibition was observed for the enzyme from *Nocardia* sp. NRRL 5646 even when benzoate concentrations were increased to 2 mM. This suggested that the *Nocardia* sp. NRRL 5646 CAR had different catalytic properties from those reported earlier [22,26].

15.1 MECHANISM OF CARBOXYLIC ACID REDUCTION BY CAR

Carboxylic acids can be chemically reduced to aldehydes, ketones, and alcohols by several methods [28]. Esters are reduced to aldehydes with diisobutylaluminum hydride (DIBAL-H). Reductions of acids and esters with lithium aluminum hydride give corresponding alcohols. Treatment of the lithium salts of acids with methyl Grignard or methyl lithium yields methyl ketones. In most of these cases, reaction conditions require temperatures below 0°C, using ether-based solvents and resulting in the aluminum salt by-products formed during reaction workup. Biocatalytic reduction of carboxylic acids would obviate many of these experimental difficulties, rendering such an important process more “green.” However, to be fully utilized and optimized, understanding the mechanism of the biocatalytic reaction was essential.

Early studies by Gross and Zenk [22,24] implicated involvement of carbonyl-AMP intermediates during enzymatic reduction of carboxylic acids. This hypothesis was based primarily on the use of radiolabeled precursors and chromatographic [thin-layer chromatography (TLC), gas chromatography (GC)] analyses of reaction mixtures to identify reaction intermediates. With *N. crassa* CAR, ^{14}C -labeled benzoyl-AMP was trapped as a hydroxamate derivative that was identified by TLC [24]. ^{14}C -Labeled benzaldehyde was chromatographically identified as the product obtained when $^{14}\text{COOH}$ -labeled benzoate was used as substrate. Although Kato et al. [26] implicated benzoyl-AMP as an intermediate in benzoic acid reduction by an enzyme from *N. asteroides*, no experimental evidence was provided.

We designed a new one-pot synthesis of benzoyl-AMP under anhydrous conditions in *N,N*-dimethylformamide. Previous syntheses of such carbonyl-AMP derivatives were relatively crude, and products had never been well defined by spectral analysis. Reaction of benzoic acid with *N,N*-carbonyldiimidazole and subsequently with 5'-adenosylmonophosphate gave the mixed anhydride in 76% isolated yield. The structure of this key intermediate in CAR reductions of benzoic acid was clearly confirmed by mass spectrometry and by proton, carbon, and phosphorous nuclear magnetic resonance (NMR) [29]. This method was also used to produce carboxy- ^{13}C -benzoyl-AMP as a labeled intermediate for NMR analysis during the enzymatic reduction. With *Nocardia* sp. NRRL 5646 CAR, ^{13}C NMR analyses of enzymatic reductions of ^{13}C -carboxy-labeled benzoic acid and its derivatives provided clear evidence for the involvement of benzoyl-AMP in the reduction. Using ^{13}C -carbonyl-tagged benzoic acid afforded well-resolved ^{13}C NMR signals for COOH (169 ppm), CO-AMP (as a trapped hydroxamate at 176.4 ppm), and CH=O (200.8 ppm). *Nocardia* sp. NRRL 5646 CAR reduction of ^{13}C -carboxy-labeled benzoic acid with ATP only (Figure 15.2a), with ^{13}C -benzoyl-AMP plus NADPH (Figure 15.2b), or with ATP plus NADPH (Figure 15.2c) gave all expected signals [30].

Benzoyl-AMP had a significantly lower (67-fold) apparent K_m ($9.66 \pm 0.71 \mu\text{M}$) and a higher (1.3-fold) V_{max} ($7.50 \pm 0.18 \mu\text{mol/min/mg}$) than those for benzoic acid [30]. These results indicated that steady-state levels of benzoyl-AMP are likely to be low during the course of the reduction of benzoic acid to benzaldehyde. The results of this study, together with our previous work, in which benzaldehyde was isolated and characterized spectrally, clearly demonstrated that the mechanism for benzoic acid reduction to benzaldehyde by purified *Nocardia* sp. NRRL 5646 CAR involved benzoyl-AMP as an intermediate [29].

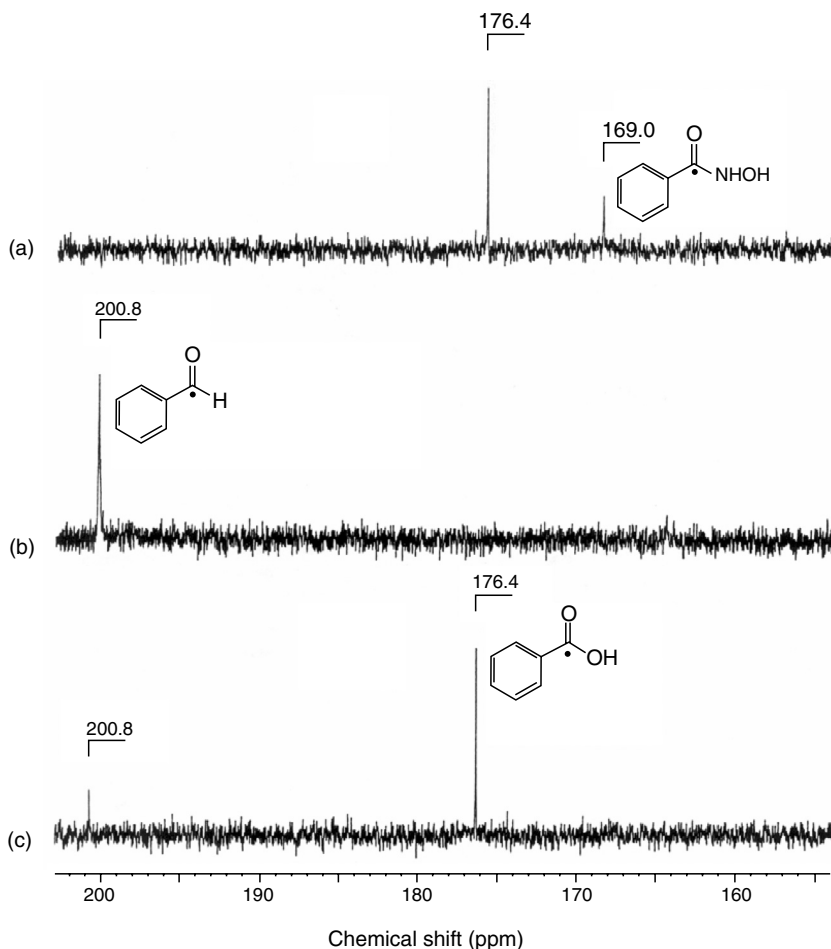


FIGURE 15.2 ^{13}C NMR study of the mechanism of benzoic acid reduction catalyzed by *Nocardia* sp. NRRL 5646 CAR. NMR spectra were obtained using incubations containing: (a) carboxy- ^{13}C -benzoic acid + ATP + NH_2OH ; (b) carboxy- ^{13}C -benzoyl-AMP + NADPH; and (c) carboxy- ^{13}C -benzoic acid + ATP + NADPH.

15.2 CAR CONTAINS A PHOSPHOPANTETHEINE ATTACHMENT SITE

CAR is produced in *Nocardia* sp., an organism that is relatively difficult to grow, and that produces limited quantities of CAR. In order to obtain reproducibly large quantities of CAR, we sought to clone and express the enzyme in a common expression host such as *Escherichia coli*. *Nocardia* sp. NRRL 5646 genomic DNA was completely digested with *Sal*I or *Acc*65I, and was then diluted fivefold, ligated, and used as the template for inverse Polymer chain reaction (PCR). Based on the N-terminal and internal sequences of purified CAR [30], the entire *Nocardia car* gene sequence was derived by inverse PCR experiments to give a polynucleotide of 6.9 kb, which included the entire *Nocardia car* gene and its flanking regions. The DNA sequence and the deduced amino acid sequence of *Nocardia car* (accession number AY495697) indicated that *Nocardia car* consisted of 3525 base pairs (bp), corresponding to a protein with 1173 amino acid residues with a calculated molecular mass of 128.3 kDa and a *pI* of 4.74. The N-terminal amino acid sequence of purified *Nocardia* CAR exactly matched the deduced amino acid sequence of the N terminus, with Ala as the first amino acid [30].

The assignment of ATG as the start codon was supported by analysis of the 5' flanking region. At 6 bp upstream from the start codon ATG lies a conserved *Streptomyces* ribosomal binding site (GGGAGG) [31,32]. *Nocardia car* gene was amplified by PCR and then cloned into plasmid pHAT10 to give plasmid pHAT-305.

The deduced amino acid sequence of *Nocardia* CAR was 60% identical to the putative acyl-coenzyme A (CoA) synthase-substrate-CoA ligase *fadD9* of *Mycobacterium tuberculosis* and *M. bovis* and was 57% identical with its *M. leprae* homolog [33]. This finding suggested that these mycobacterial proteins might function in carboxylic acid reduction, a hypothesis that remains to be tested. BLAST analysis of CAR showed an N-terminal domain (amino acids 90 to 544) with high homology to known AMP-binding proteins. The C-terminal domain (amino acids 750 to 1094) had high homology with NADPH-binding proteins.

To express rCAR, a 100 ml culture of *E. coli* [BL21(DE3) or BL21-CodonPlus(DE3)-RP] harboring pHAT-305 was grown overnight in LB-ampicillin medium. This culture was diluted 20-fold in fresh medium, and then incubated at 170 rpm on a rotary shaker at 37°C to an optical density of 0.6 at 600 nm, at which time 1 mM IPTG was added, and the induced culture was further incubated for 4.5 h. Lysate from *E. coli* BL21(DE3) and CodonPlus cells carrying pHAT-305 had moderate CAR activity (0.003 and 0.009 U/mg, respectively) compared to that of *Nocardia* wild-type cells (0.03 U/mg of protein) [30]. SDS-PAGE of cell-free extracts (CFEs) gave a Coomassie blue-stained band with an apparent molecular mass of 132.4 kDa, which was confirmed to be CAR by Western blot analysis. The DHFR-positive control and pHAT10-negative control showed the absence of a 132.4 kDa band by SDS-PAGE and Western blot analysis. A typical 1 L culture of *E. coli* BL21(DE3) CodonPlus cells carrying pHAT-305 yielded approximately 50 to 70 mg of purified CAR. Recombinant CAR (rCAR) from *E. coli* BL21-CodonPlus-(DE3)RP/pHAT-305 showed a specific activity of 0.1 U/mg, considerably less than that of wild-type CAR purified from *Nocardia* sp. NRRL 5646 (5.89 U/mg) [33].

To explain the difference in activity, we examined the CAR sequence. Buried within the deduced sequence (amino acids 683 to 693) was a serine residue (S689) and LGGxSxxA, which is the typical consensus sequence for a phosphopantetheine (Ppant) attachment site [34]. We then considered the possibility that CAR produced in wild-type *Nocardia* was actually a holo-enzyme produced by posttranslational phosphopantetheinylation of apo-CAR to afford maximal enzyme activity. A 4'-Ppant prosthetic group in active CAR could serve as a "swinging arm" that would react with acyl-AMP intermediates to form a covalently linked thioester to the C-terminal reductase domain for reduction by NADPH, aldehyde release, thiol regeneration, and a new catalytic cycle. This arrangement of the CAR protein would reflect a sequential catalytic mechanism wherein the N-terminal domain catalyzes substrate activation by formation of an initial acyl-AMP intermediate, while the C-terminal portion catalyzes the reduction of acyl-AMP by NADPH to finish a catalytic cycle (Figure 15.1). The 50-fold difference in activity between the rCAR and the wild-type CAR was attributed to the fact that the majority of the purified recombinant enzyme produced in *E. coli* was largely apo-CAR, while fully active wild-type CAR would be a phosphopantetheinylated holo-enzyme.

15.3 EVIDENCE FOR CAR PHOSPHOPANTETHEINYLATION

Phosphopantetheine transferases (Pptases) [34] are enzymes that catalyze the posttranslational modification of carrier proteins by covalently attaching the 4-Ppant moiety of CoA to a conserved serine residue (Figure 15.3). Evidence for such Pptases has been well documented in fatty acid and polyketide biosynthesis and in nonribosomal peptide synthesis [34–36]. By phosphopantetheinylation, Pptases convert inactive apo-carrier proteins to active holo-carrier

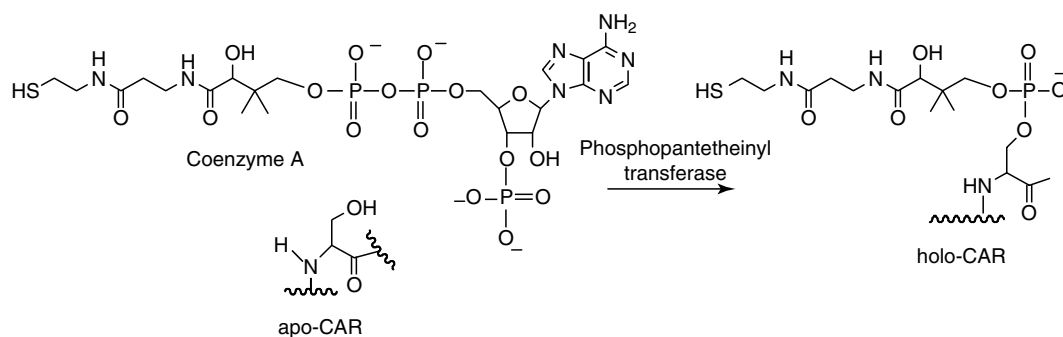


FIGURE 15.3 Phosphopantetheinylation mechanism catalyzed by Pptase.

proteins that participate in fatty acid biosynthesis, and in the biosynthesis of polyketides like bleomycin and rifamycin, bacterial acyl oligosaccharides, and acylated proteins. Because of the presence of a possible phosphopantetheinylation site in the *car* ORF, we suggested that *Nocardia* sp. NRRL 5646 contained a Pptase that converts apo-CAR to active holo-CAR [37].

Three hypotheses were thus postulated and examined:

1. Native CAR was more active because it was a phosphopantetheinylated holo-enzyme.
2. *Nocardia* contained a Pptase, which phosphopantetheinylated native apo-CAR to produce active holo-CAR.
3. rCAR was predominantly an apo-enzyme because *E. coli* lacked a Pptase that efficiently converts apo- to holo-enzyme.

If these ideas were correct, it would be possible to activate rCAR by incubating the purified rCAR “apo-enzyme” with crude *Nocardia* sp. NRRL 5646 CFE. In a typical reaction, *Nocardia* CFE (~320 to 400 µg protein) was incubated with purified rCAR (~2 nmol) in the presence of the Ppant donor CoA (1 mM) for 1 h at 28°C in a final volume of 100 µL. Enzyme activity was measured by adding this enzyme mixture (50 µL) to a solution containing 50 mM Tris buffer (pH 7.5), 1 mM EDTA, 10 mM MgCl₂, 1 mM DTT, 10% (v/v) glycerol, 1 mM ATP, 0.2 mM NADPH, and 5 mM benzoate in a final volume of 1.4 mL. The rate of change of absorbance at 340 nm was measured as a means of assessing increases in the specific activity of the enzyme preparation. The controls for this experiment were (i) CFE + rCAR, (ii) rCAR + CoA, (iii) CFE + CoA, (iv) CAR, and (v) CFE. The various combinations used with CFE, rCAR, and CoA are shown in [Table 15.1](#) [37].

Complete reaction mixtures with *Nocardia* CFE increased rCAR specific activity fivefold. The result suggests that *Nocardia* contains a Pptase that requires CoA for complete activation of CAR. Addition of CoA (1 mM) to *E. coli* BL21-CodonPlus- ((DE3)RP)/pHAT-305 cell-free lysate had no effect on the specific activity of CAR. This result confirmed our suspicion that *E. coli* expressing rCAR lacked, poorly expressed, or contained, a Pptase that was not broadly functional.

In order to ensure that the activity increase observed was due to phosphopantetheinylation of CAR by a putative Pptase in *Nocardia* sp. NRRL 5646 and not due to an artifact of the CFE, we incubated rCAR with CFE from *E. coli* JM109/pUC8-*sfp*, which contains a plasmid localized *sfp* gene encoding for the *Bacillus subtilis* Pptase Sfp. This Pptase has been used to convert apo carrier enzyme to holo form [34,36]. When rCAR was incubated with

TABLE 15.1**Specific Activity Measurements of Recombinant Carboxylic Acid Reductase (CAR) When Incubated with *Nocardia* Cell-Free Extract (CFE) and Coenzyme A (CoA) for 1 h at 28°C**

Conditions	Specific Activity (U/mg)
<i>Nocardia</i> CFE	0.01 ± 0.006
Recombinant CAR	0.3 ± 0.04
Recombinant CAR + CoA	0.3 ± 0.04
<i>Nocardia</i> CFE + CoA	0.01 ± 0.003
<i>Nocardia</i> CFE + recombinant CAR	0.57 ± 0.06
<i>Nocardia</i> CFE + recombinant CAR + CoA	1.6 ± 0.1

CFE from *E. coli* JM109/pUC8-*sfp* and CoA, a similar fivefold increase in specific activity was observed (data not shown) [37].

Benzoate induction increases the expression of wild-type CAR in *Nocardia* sp. *Nocardia* sp. NRRL 5646 CFEs obtained from cells with and without benzoate were equally effective at enhancing rCAR specific activity. This suggested that a putative *Nocardia* Pptase is constitutively expressed and may be involved in activation of other apo proteins in *Nocardia* sp. NRRL 5646 as are the “promiscuous” Pptase Sfp from *B. subtilis* [34,35] and Svp from *Streptomyces verticillus* [36].

15.4 A REVISED MECHANISM FOR HOLO-CAR REDUCTION OF CARBOXYLIC ACIDS

These results suggest that CAR reduces carboxylic acids by a more complex “swinging phosphopantetheinyl arm” process such as that illustrated in Figure 15.4 [37]. Not shown is the initial binding of both benzoic acid and ATP at the CAR N terminus where the formation of an acyl-adenylate occurs as shown in Figure 15.4a. The cysteine residue of the Ppant moiety of the holo-CAR reacts with benzoyl-AMP to form benzoyl thioester, which is covalently attached to the enzyme (Figure 15.4b). The thioester tethered to the enzyme is brought into the proximity of the C-terminal domain (Figure 15.4c) where hydride attack from NADPH reduces the thioester, yielding free benzaldehyde, NADP⁺, and a free Ppant-sulphydryl group for another catalytic cycle (Figure 15.4d).

15.5 APPLICATIONS OF CAR

Chemists continuously explore means for improving chemical processes to become both economically viable and environmentally sustainable. This involves selective catalysis, using more viable sources of raw material, and modifying synthetic procedures. The introduction of biological systems in traditional chemical synthesis provides the added advantages of catalyst versatility, regio-, chemo- and enantioselectivities, and catalysis at ambient temperature and pressure [38]. Highly selective biocatalytic reductions of carboxylic acids open new avenues for producing value-added chemicals, which are currently derived either from petroleum feedstocks or by chemical synthetic means. These range from vanillin to chiral aldehydes and alcohols obtained by kinetic resolution for the pharmaceutical, food, and agricultural industries.

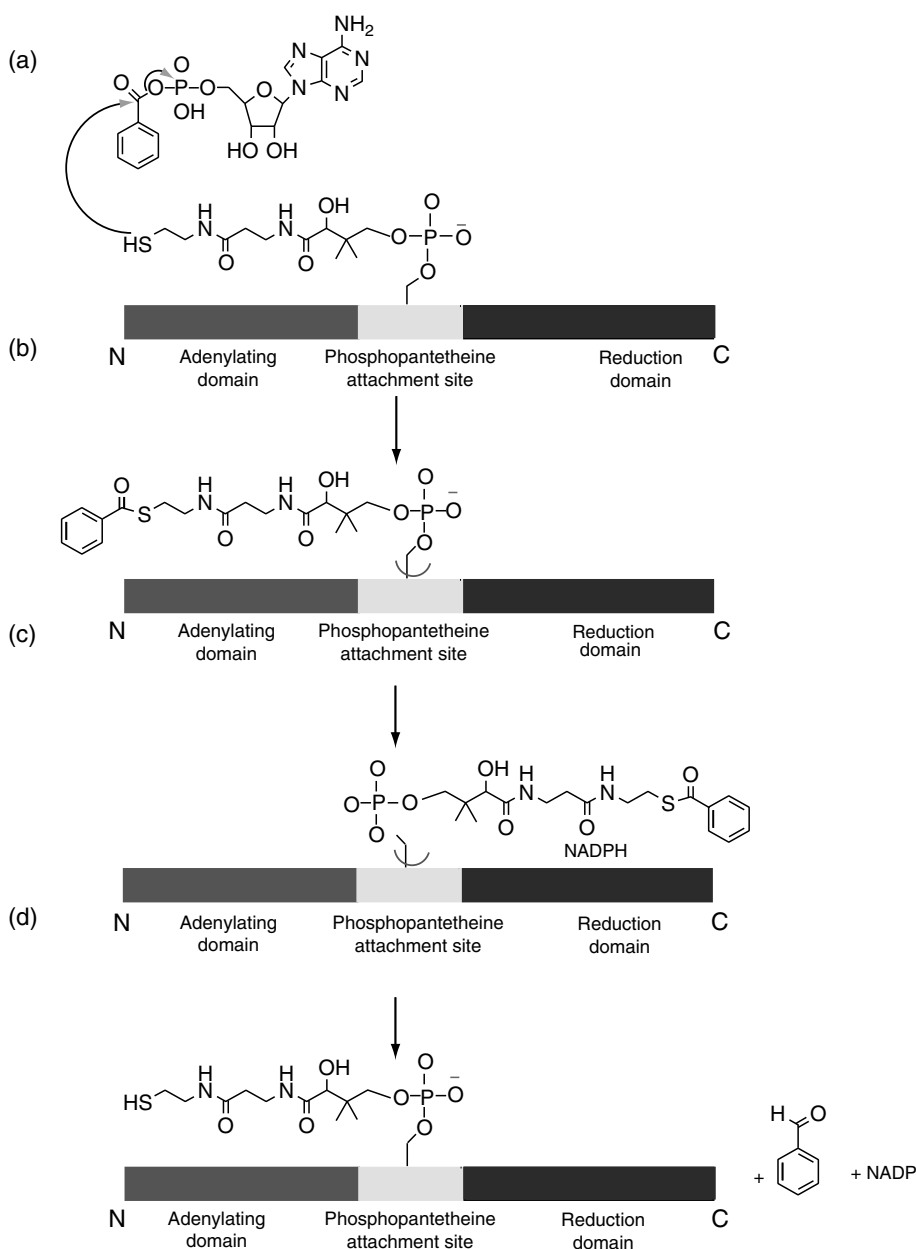


FIGURE 15.4 The catalytic cycle in holo-CAR reduction of benzoic acid to benzaldehyde.

15.6 CAR IS VERSATILE

Reduction of carboxylic acids can be accomplished by growing cultures of wild-type *Nocardia* sp. NRRL 5646 or heterologously expressed *car* in *E. coli* as well as by purified enzymes. We examined the reduction of a range of aromatic carboxylic acids and a variety of aliphatic acids. The range of oxidoreductase activities of crude *Nocardia* sp. NRRL 5646 CFE with different substrates is summarized in [Table 15.2](#). Benzoates substituted with halogens, methyl, methoxy, hydroxy, acetyl, nitro, benzoyl, phenyl, and phenoxy groups as well as aryl-ring

TABLE 15.2
Substrate Specificity of Carboxylic Acid Reductase (CAR) Obtained from Different Sources

Substrate	<i>Nocardia</i> sp. NRRL 5646	<i>Nocardia asteroides</i> JCM 3016	<i>Neurospora crassa</i>
Benzoic acid	100	100	100
2-Anisic acid	3	—	—
3-Anisic acid	87	—	53
4-Anisic acid	36	—	—
2-Chlorobenzoic acid	3	0	—
3-Chlorobenzoic acid	124	97	—
4-Chlorobenzoic acid	100	78	—
2-Hydroxybenzoic acid	0	0	—
3-Hydroxybenzoic acid	77	47	24
4-Hydroxybenzoic acid	6	16	11
2-Nitrobenzoic acid	0	0	—
3-Nitrobenzoic acid	0	22	—
4-Nitrobenzoic acid	0	0	—
2-Toluic acid	6	6	—
3-Toluic acid	4	150	—
4-Toluic acid	82	103	—

Relative rates of substrate reductions were determined by measuring the ΔOD at 340 nm as NADPH was oxidized to $NADP^+$ during the reduction reaction.

systems containing two (naphthalene) and three (fluorene) rings, and heterocyclic aromatic acids including furoic, nicotinic, and indole carboxylic acids were examined [30]. In addition, cinnamate, phenylacetate, phenylmalonate, phenylsuccinate, and 2-phenylpropionic acids were examined. Except for fluoro- and methyl-substituted benzoic acids, 3-substituted benzoic acids (bromo, chloro, hydroxyl, methoxyl) were the best substrates within their respective aryl-carboxylic acid series. The range of aromatic substrates used by CAR is similar to that observed by Kato et al. [26]. In general, *ortho*-substituted benzoates were the poorest substrates from within any of the substrates compared. The CFE from *Nocardia* efficiently reduced naphthoic acids, but only reduced indole-3- and indole-5-carboxylic acids. In addition, CFEs reduced furoic acids, nicotinic acid and phenylmalonate, phenylsuccinate, phenylacetate and phenylpropionate, albeit at slower rates than benzoate itself. Of all the compounds examined, the best substrates were benzoic acid, 3-bromobenzoic acid, 3-chlorobenzoic acid, 4-fluorobenzoic acid, 4-methylbenzoic acid, 3-methoxy-benzoic acid, and 2-naphthoic acid. Nitrobenzoates were not reduced at all.

rCAR also reduced a number of natural mono-, di-, and tricarboxylic acids (Table 15.3) [37]. Rates of reduction of D-tartaric acid, α -ketoglutaric acid, and *cis*- and *trans*-aconitic, citric, and malic acids were higher than those for benzoic acid. Regiospecificity in carboxylic acid reduction remains to be established for citric, tartaric, and malic acids. rCAR in D-tartaric acid specifically offered the possibility of resolving tartaric acid racemates. Pyruvic, isocitric, fumaric, and maleic acids were not substrates, indicating that carboxyl moieties adjacent to sp^2 carbon centers might be less susceptible to rCAR reduction.

15.7 CAR IS STEREOSELECTIVE

The 2-arylpropionic acid derivative ibuprofen (**1**, Figure 15.5) is a potent, oral nonsteroidal antiinflammatory, antipyretic, and analgesic agent [39,40]. Pharmacological activities of

TABLE 15.3
Substrate Specificity of rCAR

Substrate	Relative Activity
Benzoic acid	100
<i>cis</i> -Aconitic acid	130
<i>trans</i> -Aconitic acid	130
Citric acid	120
Fumaric acid	0
3-Hydroxy-3-methylglutaric acid	40
Isocitric acid	0
2-Ketoglutaric acid	160
DL-Lactic acid	50
L-Lactic acid	50
Maleic acid	0
DL-Malic acid	105
D-Malic acid	105
L-Malic acid	130
Oxaloacetic acid	80
Pyruvic acid	0
D-Tartaric acid	120
L-Tartaric acid	0
Tricarballic acid	80

Relative rates of substrate reductions were determined by measuring the ΔOD at 340 nm as NADPH was oxidized to $NADP^+$ during the reduction reaction.

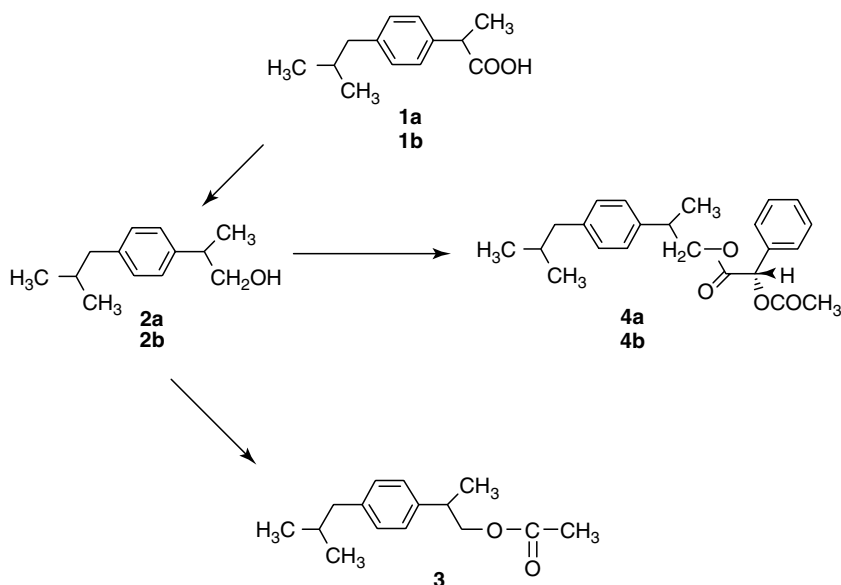


FIGURE 15.5 Schemes for ibuprofen, metabolites, and derivatives: **1a**, racemic ibuprofen; **1b**, *S*(+)-ibuprofen; **2a**, racemic ibuprofenol; **2b**, *S*(+) ibuprofenol **3**, ibuprofen acetate; **4a**, *S*(+)-*O*-acetylmandelate ester of compound **2a**; **4b**, *S*(+)-*O*-acetylmandelate ester of **2b**.

ibuprofen reside almost exclusively in its *S*-enantiomer [41]. Ibuprofen was an attractive substrate candidate for investigation because of its overall importance as a widely used drug, and the opportunity to examine the possible stereoselective properties of the CAR reaction.

With growing cultures of *Nocardia* sp. NRRL 5646 two major metabolites of racemic ibuprofen were formed, isolated, and identified as ibuprofenol (**2**) and the corresponding acetate derivative (**3**) by spectral methods (Figure 15.5) [20]. An unoptimized preparative scale reaction conducted with (+/–)-ibuprofen was stopped at 24 h when TLC analysis indicated that approximately 50% of the substrate had been converted into other products. Unreacted and recovered ibuprofen was largely *S*(+)-ibuprofen (**1b**, Figure 15.5). The enantiomeric excess (ee) of *R*(+)-ibuprofenol obtained from the biotransformation of racemic ibuprofen was determined to be 61.2% by NMR spectral analysis of the *S*(+)-*O*-acetylmandelate ester (Figure 15.6). This finding showed that whole-cell *Nocardia* sp. NRRL 5646 reactions were *R*(–)-selective with (+/–)-ibuprofen (**1a**, Figure 15.5), and offered the possibility that other racemic carboxylic acids can be resolved.

The availability of pure CAR from *Nocardia* sp. NRRL 5646 allowed the evaluation of enantioselectivity of the reduction of ibuprofen isomers [30]. The K_m and V_{max} , respectively, for (*S*)-(+)-ibuprofen were $155 \pm 18 \mu\text{M}$ and $0.148 \pm 0.003 \mu\text{mol/min/mg}$. By comparing V_{max}/K_m ratios of carboxylic acid reduction for the two isomers, the enantiomeric ratio was calculated to be 40.4 for the (*R*)-(–)-isomer over the (*S*)-(+)-isomer [42]. Using these data, theoretically, the reduction of ibuprofen by the pure enzyme should give an ee of 86.9% at 50% conversion yield [42]. This compares favorably with the observed ee of 61.2% for the isolated and unoptimized reduction of racemic ibuprofen [20].

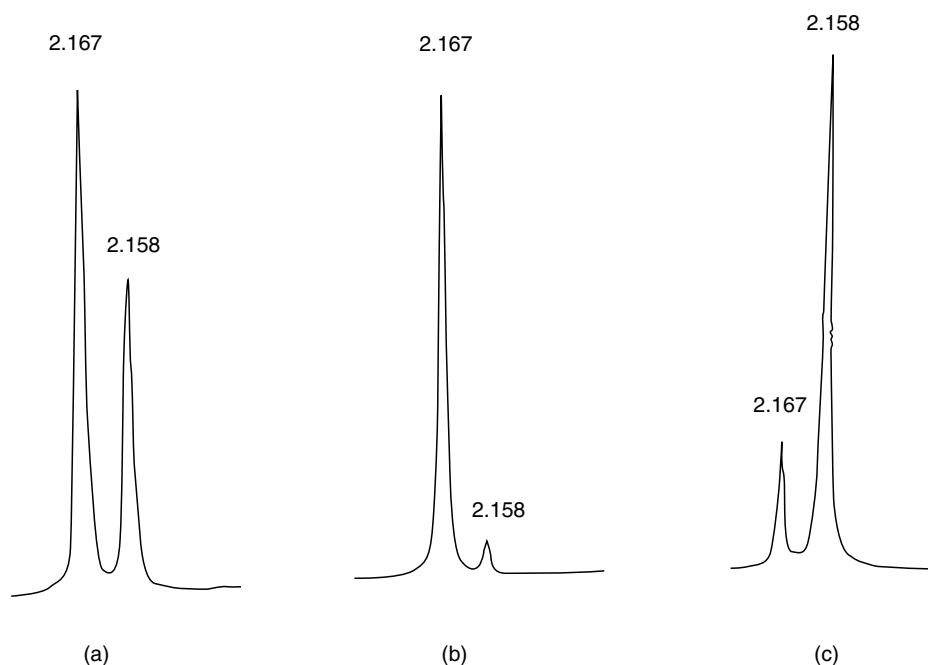


FIGURE 15.6 ^1H NMR signals of 600 Mhz, for the acetyl esters of **4a** *S*(+)-*O*-acetylmandelate ester of: (a) racemic ibuprofenol; (b) *S*(–)-ibuprofenol; and (c) *R*(+)-ibuprofenol from microbial transformation of racemic ibuprofen.

While it may appear disadvantageous for whole cells to reduce carboxylic acids to aldehydes, alcohols, and further esterify them to the corresponding acetates, such a combination of reactions may be highly desirable, for example, in the conversion of carboxyl substrates into esters of value or fragrances and flavors.

15.8 SYNTHESIS OF VALUE-ADDED CHEMICALS

15.8.1 VANILLIN

Natural vanilla obtained from the dried pods of the orchid *Vanilla planifolia* accounts for only 0.2% of the world flavor market (20 t/y out of 1.2×10^5 t/y) and the remainder is supplemented by chemical synthesis of vanillin (**6**) from guaiacol (**8**) [43]. Vanillin has been produced by microbial transformation from natural substrates, including phenolic stilbenes [44,45], eugenol [46], and ferulic acid [47,48].

In whole-cell *Nocardia* biotransformation reactions, vanillic acid (**5**) decarboxylation to guaiacol (**8**) was the major complicating pathway [49,50]. The identification of guaiacol (**8**) and vanillyl alcohol (**7**) as metabolites confirmed that *Nocardia* sp. strain NRRL 5646 possesses two different metabolic pathways for the biotransformation of vanillic acid (**5**). These pathways are (i) decarboxylation to guaiacol (**8**) and (ii) reduction to vanillin (**6**) and its subsequent reduction to vanillyl alcohol (**7**) (Figure 15.7).

With pure *Nocardia* CAR, the ATP- and NADPH-dependent reduction of vanillic acid was quantitative, yielding only vanillin and no complicating by-products [51]. In 24 h *in vitro* reactions containing 0.1 mmol of substrate and rCAR, preparative TLC gave 7.5 mg of vanillin (48% yield). The difference between the yield in the *in vitro* reaction of pure *Nocardia* CAR and rCAR may be due to the differences in their specific activities [33]. With *in vivo* biotransformations of vanillin with *E. coli* BL21-CodonPlus(DE3)-RP harboring plasmid pHAT-305, vanillin was observed at 2 h and was converted to the corresponding alcohols in 24 h [33].

15.8.2 FERULIC ACID

Ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-propenoic acid] (**9**) is an extremely abundant plant product available from corn kernel hulls obtained from wet milling [52,53]. Vanillic acid

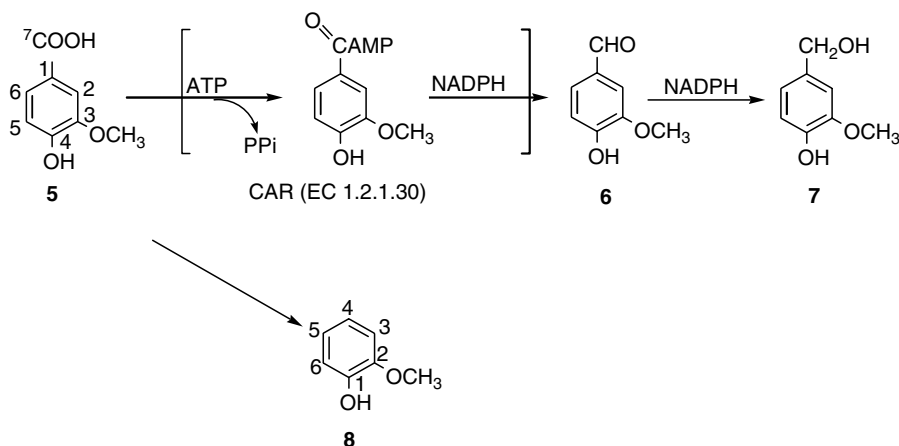


FIGURE 15.7 Whole-cell and enzymatic biotransformation of vanillic acid.

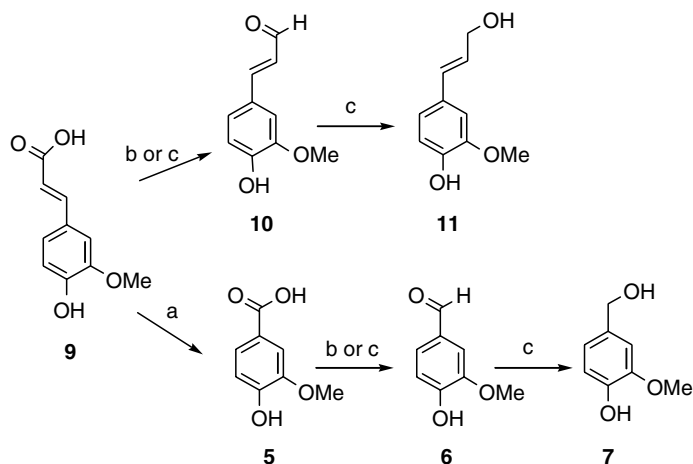


FIGURE 15.8 Reduction of ferulic acid to value-added chemicals: (a) microbial oxidation by strains of *Bacillus*, *Pseudomonas*, *Polyporus*, *Rhodotorula*, or *Streptomyces*; (b) Purified recombinant CAR; (c) *E. coli* BL21-CodonPlus-((DE3)RP)/pHAT-305.

is the major product obtained by oxidation of ferulic acid by species of *Bacillus* [54], *Pseudomonas* [55,56], *Polyporus* [57], *Rhodotorula* [58], and *Streptomyces* [59]. Thus, vanillic acid is an abundant, readily available precursor for the biocatalytic synthesis of vanillin using the AAOR. So in a two-step/single-pot process, one can envision a simple route to the preparation of vanillin from ferulic acid (Figure 15.8).

In vitro reaction of ferulic acid with rCAR afforded a smooth reduction of ferulic acid to coniferyl aldehyde (**10**) [33]. The current market price of coniferyl aldehyde is approximately \$8,000/lb. Incubation of ferulic acid with growing cultures of *E. coli* BL21-CodonPlus-((DE3)RP)/pHAT-305 expressing CAR led to the production of coniferyl alcohol (**11**). The current market price of coniferyl alcohol is approximately \$109,000/lb. Such reductions of the carboxyl moiety of ferulic acid to its aldehyde and alcohol were not observed when ferulic acid was incubated with resting cells of *Nocardia* sp. NRRL 5646.

15.9 CONCLUSIONS

The heterologous expression of CAR in *E. coli* provides a new avenue for the production of value-added chemical like vanillin, coniferyl aldehyde, and their alcohols that have a large interest in the pharmaceutical, food, and agricultural industries. Biotransformation reactions using IPTG-induced whole *E. coli* BL21(DE3)CodonPlus cells harboring plasmid pHAT-305 were simple to conduct and resulted in the smooth conversion of carboxylic acids to aldehydes and subsequently to alcohols. With whole cells, expensive cofactors are not necessary [33,51], rendering the biocatalytic reaction more practical on large scales. Reduction of aldehydes formed by CAR to alcohols by an endogenous *E. coli* alcohol dehydrogenase similar to that observed in *Nocardia* [51] is relatively slow. Biochemical engineering approaches with the recombinant organism can be exploited to diminish this unwanted side reaction.

The unique *car* sequence for the CAR enzyme may be used to produce recombinant *E. coli* cultures which can be grown easily for direct use in whole-cell biocatalytic conversions of natural or synthetic carboxylic acids [30,60]. Alternatively, this gene sequence or its homologs

may be incorporated into the genomes of multiple recombinant strains through pathway engineering to be used in combinatorial biocatalytic syntheses of useful compounds.

ACKNOWLEDGMENTS

This work was supported by the Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture, under Agreement Nos. 2002-34188-12035 and 2004-34188-15067. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Agriculture. We gratefully acknowledge the scientific contributions to this work made by Drs. Yijun Chen, Tao Li, and Aimin He.

REFERENCES

1. Davies, H.G. et al., *Biotransformations in Preparative Organic Chemistry: the Use of Isolated Enzymes and Whole Cell Systems in Synthesis*, Academic Press, New York, 1989.
2. Hanc, O.F., The microbial transformation of organic compounds, *J. Ind. Chem. Soc.*, 52, 4–16, 1975.
3. Jones, J.B., Perlman, D., and Sih, C.J., Eds., *Applications of Biochemical Systems in Preparative Organic Chemistry*, vols. I and II, Wiley, New York, 1976.
4. Kieslich, K., Preparative anwendbare mikrobiologische reaktionen. zweiter teil. reduktionen, hydrolytische reaktionen, acylierungen, und kondensationen, aminierungen, decarboxylierungen, *Synthesis*, 1, 147, 1969.
5. Leuenberger, H.G.W., Biotransformation: a useful tool in organic chemistry, *Pure Appl. Chem.*, 62, 753, 1990.
6. Porter, R. and Clark, S., Eds., *Enzymes in Organic Synthesis*, CIBA Foundation Symposium III, Pitman Publishers, London, 1985.
7. Roberts, S.M. and Wiggins, D.K., *Preparative Transformations: Whole Cell and Isolated Enzymes in Organic Synthesis. A Core Manual*, Wiley, New York, 1992.
8. Liu, W.G. et al., Stereochemistry of microbiological hydroxylations of 1,4-cineoles, *J. Org. Chem.*, 53, 5700, 1988.
9. Sariaslani, F.S. and Rosazza, J.P.N., Biocatalysis in natural products chemistry, *Enzyme. Microb. Technol.*, 6, 242, 1984.
10. Sih, C.J. and Abushanab, E., Biochemical procedures in organic synthesis, *Ann. Rep. Med. Chem.*, 12, 298, 1977.
11. Sih, C.J. and Rosazza, J.P.N., Microbial transformations in organic synthesis, Chap. III, in *Applications of Biochemical Systems in Organic Chemistry*, Part I, Jones, J.B., Ed., Wiley, New York, 1976, p. 69.
12. Ward, O.P. and Young, C.S., Reductive biotransformations of organic compounds by cells or enzymes of yeast, *Enzyme. Microb. Technol.*, 12, 482, 1990.
13. Jezo, I. and Zemek, J., Enzymatische reduktion einiger aromatischer carboxysäuren, *Chem. Pap.*, 40(2), 279, 1986.
14. White, H. et al., Carboxylic acid reductase, a new tungsten enzyme catalyses the reduction of non-activated carboxylic acids to aldehydes, *Eur. J. Biochem.*, 184, 89, 1989.
15. Arfman, H.A. and Abraham, W.R., Microbial reduction of aromatic carboxylic acids, *Z. Naturforsch.*, 48(c), 52, 1993.
16. Raman, T.S. and Shanmugasundaram, E.R.B., Metabolism of some aromatic acids by *Aspergillus niger*, *J. Bacteriol.*, 84, 1340, 1962.
17. Bachman, D.M., Dragoon, B., and John, S., Reduction of salicylate to saligenin by *Neurospora*, *Arch. Biochem. Biophys.*, 91, 326, 1960.
18. Tsuda, Y., Kawai, K., and Nakajima, S., Asymmetric reduction of 2-methyl-2-aryloxyacetic acids by *Glomerella cingulata*, *Agric. Biol. Chem.*, 48(5), 1373, 1984.

19. Tsuda, Y., Kawai, K., and Nakajima, S., Microbial reduction of 2-phenylpropionic acid, 2-benzyloxypropionic acid and 2-(2-furfuryl)propionic acid, *Chem. Pharm. Bull.*, 33(11), 4657, 1985.
20. Chen, Y. and Rosazza, J.P.N., Microbial transformation of ibuprofen by a *Nocardia* species, *Appl. Environ. Microbiol.*, 60(4), 1292, 1994.
21. Kato, N. et al., Microbial reduction of benzoate to benzyl alcohol, *Agric. Biol. Chem.*, 52(7), 1885, 1988.
22. Gross, G.G. and Zenk, M.H., Reduktion aromatischer säuren zu aldehyden und alkoholen im zellfreien system. 1. Reinigung und eigenschaften von aryl-aldehyde, NADP-oxidoreduktase aus *Neurospora crassa*, *Eur. J. Biochem.*, 8, 413, 1969.
23. Gross, G.G. and Zenk, M.H., Reduktion aromatischer säuren zu aldehyden und alkoholen im zellfreien system. 2. Reinigung und eigenschaften von aryl-alkohol, NADP-oxidoreduktase aus *Neurospora crassa*, *Eur. J. Biochem.*, 8, 420, 1969.
24. Gross, G.G., Formation and reduction of intermediate acyl-adenylate by aryl-aldehyde NADP oxidoreductase from *Neurospora crassa*, *Eur. J. Biochem.*, 31, 585, 1972.
25. Hempel, J., Nicholas, H. and Lindahl, R., Aldehyde dehydrogenases: widespread structural and functional diversity within a shared framework, *Protein Sci*, 2(11), 1890, 1993.
26. Kato, N. et al., Purification and characterization of aromatic acid reductase from *Nocardia asteroides* JCM 3016, *Agric. Biol. Chem.*, 55(3), 757, 1991.
27. Clonis, Y.D., Dye-ligand chromatography, in *Reactive Dyes in Protein and Enzyme Technology*, Clonis, Y.D. et al., Eds., Stockton Press, New York, 1989, p. 33.
28. Smith, M.B. and March, J., *Advanced Organic Chemistry*, 5th ed., Wiley, New York, 2001, p. 532.
29. Li, T. and Rosazza, J.P.N., NMR identification of an acyl-adenylate intermediate in the aryl-aldehyde oxidoreductase catalyzed reaction, *J. Biol. Chem.*, 273, 34230, 1998.
30. Li, T. and Rosazza, J.P.N., Purification, characterization, and properties of an aryl aldehyde oxidoreductase from *Nocardia* sp. strain NRRL 5646, *J. Bacteriol.*, 179, 3482, 1997.
31. Mulder, M.A., Zappe, H., and Steyn, L.M., Mycobacterial promoters, *Tuber. Lung Dis.*, 78, 211, 1997.
32. Strohl, W.R., Compilation and analysis of DNA sequences associated with apparent streptomycete promoters, *Nucleic Acids Res.*, 20, 961, 1992.
33. He, A. et al., *Nocardia* sp. carboxylic acid reductase, cloning, expression, and characterization of a new aldehyde oxidoreductase family, *Appl. Environ. Microbiol.*, 70, 1874, 2004.
34. Lambalot, R.H. et al., A new enzyme superfamily: the phosphopantetheinyl transferases, *Chem. Biol.*, 3, 923, 1996.
35. Sanchez, C. et al., Identification and characterization of a type II peptidyl carrier protein from the bleomycin producer *Streptomyces verticillus* ATCC 15003, *Chem. Biol.*, 8, 725, 2001.
36. Quadri, L.E. et al., Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases, *Biochemistry*, 37, 1585, 1998.
37. Venkitasubramanian, P. and Rosazza, J.P.N., unpublished results, 2006.
38. Thomas S.M., DiCosimo, R., and Nagarajan, V., Biocatalysis, applications and potentials for the chemical industry, *Trends Biotechnol.*, 20(6), 238, 2002.
39. Simon, L. and Mills, J., Non-steroidal anti-inflammatory drugs, *New Engl. J. Med.*, 302, 1179, 1980.
40. Kantor, T.G., Ibuprofen, *Ann. Intern. Med.*, 91, 877, 1979.
41. Geissinger, G. et al., Pharmacological differences between *R*(-) and *S*(+)ibuprofen, *Agents Actions*, 27, 455, 1989.
42. Chen, C.S. et al., Quantitative analysis of biochemical kinetic resolutions of enantiomers, *J. Am. Chem. Soc.*, 104, 7294, 1982.
43. Esposito, L. et al., in *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed., Kroschwitz, J. I. and Howe-Grant, M., Eds., Wiley, New York, vol. 24, 1997, p. 812.
44. Clark, G.S., Vanillin, *Perfum. Flavor.*, 15, 45, 1990.
45. Priefert, H., Rabenhorst, J., and Steinbüchel, A., Biotechnological production of vanillin, *Appl. Microbiol. Biotechnol.*, 56, 296, 2001.
46. Yoshimoto, T. et al., Dioxxygenase for styrene cleavage manufactured by *Pseudomonas*, Jap. Patent No. 2,195,871, 1990.
47. Washisu, Y. et al., Manufacture of vanillin and related compounds with *Pseudomonas*, Jap. Patent No. 5,227,980, 1993.

48. Rabenhorst, J. and Hopp, R., Process for the preparation of vanillin, U.S. Patent 5,017,388, 1991.
49. Mulheim, A. and Lerch, K., Towards a high-yield bioconversion of ferulic acid to vanillin, *Appl. Microbiol. Biotechnol.*, 51, 456, 1999.
50. Labuda, I.M., Goers, S.K., and Keon, K.A., Bioconversion process for the production of vanillin, U.S. Patent 5,128,253, 1992.
51. Li, T. and Rosazza, J.P.N., Biocatalytic synthesis of vanillin, *Appl. Environ. Microbiol.*, 66, 684, 2000.
52. Huang, Z., Dostal, L., and Rosazza, J.P.N., Mechanisms of ferulic acid conversions to vanillic acid and guaiacol by *Rhodotorula rubra*, *J. Biol. Chem.*, 268, 23954, 1993.
53. Rosazza, J.P.N. et. al., Biocatalytic transformation of ferulic acid, an abundant aromatic natural product, *J. Ind. Microbiol.*, 15, 457, 1995.
54. Gurujeyalakshmi, G. and Mahadevan, A., Dissimilation of ferulic acid by *Bacillus subtilis*, *Curr. Microbiol.*, 16, 69, 1987.
55. Jurková, M. and Wurst, M., Biodegradation of aromatic carboxylic acids by *Pseudomonas mira*, *FEMS Microbiol. Lett.*, 111, 245, 1993.
56. Toms, A. and Wood, J.M., The degradation of *trans*-ferulic acid by *Pseudomonas acidovorans*, *Biochemistry*, 9, 337, 1970.
57. Ishikawa, H., Schubert, W.J., and Nord, F.F., The degradation by *Polyporus versicolor* and *Formes fomentarius* of aromatic compounds structurally related to softwood lignin, *Archiv. Biochem. Biophys.*, 100, 140, 1970.
58. Mulheim, A. and Lerch, K., Towards a high-yield bioconversion of ferulic acid to vanillin, *Appl. Microbiol. Biotechnol.*, 51, 456, 1999.
59. Sutherland, J.B., Crawford, D.L., and Pometto, A.L. III, Metabolism of cinnamic, *p*-coumaric, and ferulic acids by *Streptomyces setonii*, *Can. J. Microbiol.*, 29, 1253, 1983.
60. Rosazza, J.P.N. and Li, T., Purification characterization and properties of an aryl-aldehyde oxidoreductase from *Nocardia* sp. strain NRRL 5646. U.S. Patent 579,559, 1998.

16 Dehalogenases in Biodegradation and Biocatalysis

Dick B. Janssen

CONTENTS

16.1	Introduction	441
16.2	Haloalkane Dehalogenases	445
16.2.1	Properties and Catalytic Mechanism	445
16.2.2	Environmental Relevance	448
16.2.3	Protein Engineering Studies	448
16.2.4	Conversion of 1,2,3-Trichloropropane	449
16.2.5	Enantioselectivity	450
16.2.6	Immobilized Haloalkane Dehalogenases	450
16.3	Haloacid Dehalogenases	451
16.3.1	Properties of L-2-Chloropropionic Acid Dehalogenases	451
16.3.2	D-Specific Chloropropionic Acid Dehalogenase	452
16.3.3	Production of Optically Active Chloropropionic Acid	452
16.4	Halohydrin Dehalogenase	452
16.4.1	Properties	452
16.4.2	Biocatalytic Potential of Halohydrin Dehalogenation	454
16.4.3	Biocatalytic Ring Opening of Epoxides	455
16.4.4	Other Dehalogenases That Convert Halohydrins	458
16.5	Conclusions and Outlook	458
	References	459

16.1 INTRODUCTION

The first descriptions of dehalogenases date back to the 1960s. Davies and Evans [1] and Goldman [2] described enzymes that dehalogenate fluoroacetate, and Castro and Bartnicki [3] studied an enzyme that releases bromide from 2,3-dibromo-1-propanol. Dehalogenases were initially classified on the basis of their substrate range. However, genetic analysis has revealed that dehalogenases acting on similar substrates (e.g., fluoroacetate and chloroacetate) may belong to unrelated protein families, whereas dehalogenases that act on rather different substrates (e.g., fluoroacetate and 1,2-dibromoethane) may belong to the same phylogenetic superfamily. Fluoroacetate, 1,2-dichloroethane, 1,3-dichloropropene, and tetrachlorocyclohexadiene are dehalogenated by dehalogenases that have low sequence similarity, but all four belong to the α/β -hydrolase fold family. Most 2-chlorocarboxylic acid dehalogenases that

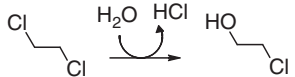
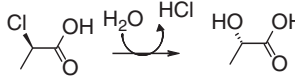

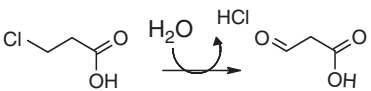
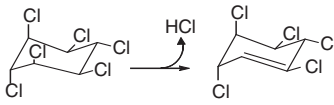
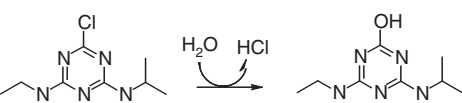
have been studied belong to the HAD superfamily of dehalogenases–phosphatases (Table 16.1, L-DEX, DhlB) [4], but there are also haloacid dehalogenases such as the DL-2-haloacid dehalogenase from *Pseudomonas* sp. 113 [5], that are not related to the HAD superfamily, and form a separate group of enzymes, some of which are specific for D-chloropropionic acid. The phylogenetic classification, as used in Table 16.1, is now replacing the old substrate-based classification in most studies, and the majority of haloacid dehalogenases can be grouped into two families [4].

The reaction types catalyzed by dehalogenases and the cofactors that these enzymes use are highly diverse (Table 16.1). Several groups of dehalogenases catalyze a simple hydrolysis reaction without the use of cofactors, prosthetic groups, or metal ions. This includes haloalkane dehalogenases and haloacid dehalogenases. Other dehalogenases, especially those acting on halogens bound to an aromatic ring, have completely different mechanisms, often involving cofactors such as glutathione or NADPH. For example, the dehalogenation of 4-chlorobenzoic acid occurs after activation by coupling of the compound to coenzyme A (CoA) and is catalyzed by a dehalogenase (Table 16.1, CbzA) that produces 4-hydroxybenzoyl CoA through a covalent substrate–enzyme intermediate. The CoA is released by a thiolase after dechlorination. The first step in the pentachlorophenol biodegradation pathway is catalyzed by an NADPH-dependent monooxygenase (Table 16.1, PcpC) that removes a chlorine at the para-position, whereas a reductive glutathione-dependent enzyme is involved in the second dechlorination step, in which tetrachlorohydroquinone is the substrate.

Reductive dehalogenases (e.g., PceA in Table 16.1) use an external electron donor, and replace a halogen substituent by hydrogen, releasing halide. The reductive dehalogenases have drawn considerable attention because of their role in the reductive dechlorination of notorious environmental pollutants such as PCBs, chlorobenzenes, and especially chloroethenes. Tetrachloroethene, trichloroethene, and other chloroethenes can be sequentially dechlorinated by dehalogenases that are produced by anaerobic bacteria, which can use chlorinated compounds as their physiological electron acceptor. The bacterium *Dehalococcoides ethenogenes*, which can reductively dechlorinate tetrachloroethene all the way to ethene, possesses 17 putative reductive dehalogenase genes. These genes show the presence of two iron–sulfur cluster binding motifs that also occur in ferredoxins, as well as conserved tryptophan and histidine residues that may play a role in catalysis, but it is not known how the cobalamin cofactor that is involved in dechlorination is bound to the enzymes. No structures are known for these important proteins [6].

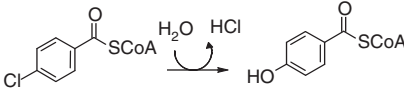
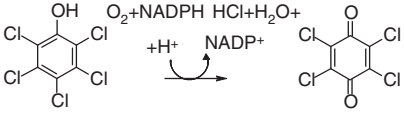
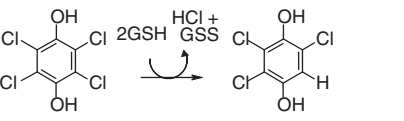
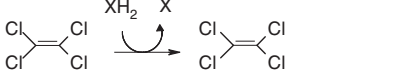
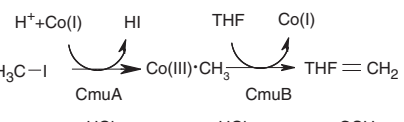
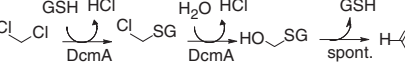
An intriguing characteristic of many dehalogenases is their capacity to bind and convert xenobiotic compounds that have a structure which does not naturally occur in the biosphere. Although thousands of naturally occurring organohalogenes have been identified, including some structures that were rather unexpected, such as chloroethenes [7], it is likely that many of the compounds that have been the subject of detailed biodegradation studies did not occur on earth in biologically significant concentrations before the onset of their industrial production some 100 years ago. Thus, compounds such as 1,2-dichloroethane, tetrachloroethylene, 1,3-dichloropropylene, epichlorohydrin, hexachlorocyclohexane, and pentachlorophenol only entered the biosphere and challenged microbial evolution after they were synthesized and applied by man. This raises the question whether the dehalogenases that are now found to convert these xenobiotics already existed in preindustrial times, in which case they probably have played a role in the metabolism of natural compounds that carry a halogen substituent or a substituent that is biochemically similar to a halogen group, or whether they evolved by short evolutionary pathways from preindustrial enzymes that acted on unidentified natural compounds. In the latter case, the evolutionary predecessor could again have been a dehalogenase that converts a natural organohalogen, or an enzyme that acts on a nonhalogenated compound and only acquired dehalogenase activity after some mutations.

TABLE 16.1**Diversity of Dehalogenases, Their Reaction Types, Phylogenetic Grouping and Key Mechanistic Features**

Type (example)	Reaction with Xenobiotic Compound	Family/Cofactor	Mechanism
Haloalkane dehalogenase (DhlA, DhaA, LinB)		α,β -Hydrolase fold, similar to lipases	Catalytic triad, Asp as nucleophile, distinct halide-binding site, covalent intermediate [16, 17]
Halocarboxylic acid dehalogenase (L-DEX, DhlB)		Haloacid dehalogenase–phosphatase fold (HAD family)	Catalytic Asp close to N terminus as the nucleophile, covalent intermediate [47, 48]
Halohydrin dehalogenase (HheA, HheB, HheC)		Short-chain dehydrogenase/reductase family (SDR proteins)	Catalytic triad for H ⁺ abstraction, halide binding site, noncovalent catalysis [56]
Chloroacrylic acid dehalogenase (CaaD)		4-Oxalocrotonate tautomerase family (trimers or hexamers)	N-terminal nucleophilic Pro, no covalent intermediate, hydratase-like mechanism [58]
Hexachlorocyclohexane dechlorinase (LinA)		Scytalone dehydratase	Abstraction of an axial proton, with concomitant anti-elimination of a transaxial chloride from the adjacent carbon atom [80, 81]
Atrazine chlorohydrolase (AtzA)		Amidohydrolase superfamily	Activation by metal group [10]

continued

TABLE 16.1 (continued)**Diversity of Dehalogenases, Their Reaction Types, Phylogenetic Grouping and Key Mechanistic Features**

Type (example)	Reaction with Xenobiotic Compound	Family/Cofactor	Mechanism
4-Chlorobenzoyl-CoA dehalogenase (CbzA)		Enoyl hydratase superfamily	Asp as nucleophile, covalent intermediate [82]
Pentachlorophenol dehalogenase (PcpA)		NADPH-dependent flavin monooxygenase, para-hydroxybenzoate hydroxylase group	Oxygen substitution at the para-position, elimination of halide from unstable product [83]
Tetrachlorohydroquinone reductive dehalogenase (PcpC)		Zeta class of the glutathione S-transferase superfamily	Nucleophilic attack of GSH on substrate, displacement of GSH by Cys to form a GS-Cys heterodisulfide, release of GSH by second GSH [84, 85]
Tetrachloroethene reductive dehalogenase (PceA)		Reductive dechlorination, cobalamin-containing enzyme	Either formation of organocobalt adduct or electron donation by corrinoid cofactor [6]
Methyltransferase I (CmuA), methyltransferase II (CmuB)		Methyltransferase domain fused to corrinoid-binding domain (CmuA), methyl transferase-like protein (CmuB)	Methyl group transfer, nucleophilic substitution of chlorine by Co [86]
Dichloromethane dehalogenase (DcmA)		Theta class of the glutathione S-transferase superfamily	Glutathione activation by Tyr, decomposition of adduct [87]

Evolutionary and mechanistic studies have indicated that both these options may occur, although data are very scarce. The *Xanthobacter autotrophicus* haloalkane dehalogenase (DhlA, Table 16.1) that converts 1,2-dichloroethane was proposed to have evolved from a debrominating enzyme by a few mutations in the cap domain that influence the activity, including generation of short tandem repeats that are found both in the wild-type enzyme and in derivatives with an enhanced rate of 1-chlorohexane hydrolysis [8,9]. Atrazine chlorohydrolase from *Pseudomonas* sp. strain adenosine diphosphate (ADP) (AtzA) may have evolved from a protein that acts on an amine-substituted triazine [10]. The enzyme, which acts as a hydrolytic dehalogenase and belongs to the amidohydrolase superfamily, differs by only nine amino acids in its sequence from melamine (2,4,6-triamino-1,3,5-triazine) deaminase (TriA), and just a few mutations suffice to switch the activity between deaminase and chlorohydrolase [11]. Another member of the amidohydrolase superfamily, AtzB, can catalyze both deamination and dechlorination of triazine derivatives, indicating that deamination and dechlorination indeed are mechanistically similar and can be catalyzed by the same protein scaffold.

Most dehalogenases appear to belong to protein superfamilies that mainly consist of enzymes that catalyze reactions with natural substrates. Examples are the HAD, the α/β -hydrolase fold, and the short-chain dehydrogenase/reductase (SDR) superfamilies, which are discussed in more detail later. A further example of such a superfamily that encompasses some dehalogenases is the 4-OT group of isomerases–tautomerases, of which 4-oxalocrotonate tautomerase is the classical example [12]. This group of trimeric or hexameric enzymes includes *cis*- and *trans*-3-chloroacrylic acid dehalogenases (CaaD, Table 16.1) [13]. *trans*-3-Chloroacrylic acid is remarkably easy to dehalogenate enzymatically in view of its extreme stability at high pH and temperature [14]. Apparently, the substrate and water are very effectively labilized along the reaction coordinate. This allows a reaction that, mechanistically, is addition of water to the double bond, which is catalyzed by the conserved N-terminal proline that deprotonates water to attack C3 and donates a proton to C2 [15].

Research on dehalogenases has been inspired by the possibility that they can play a role in the bioremediation of contaminated groundwater or wastewater and the certainty that they are important for biodegradation of halogenated pollutants in the natural environment. Dehalogenases appear to be attractive detoxification catalysts as they carry out the most critical reaction in the metabolism of halogenated organics: the cleavage of carbon–halogen bonds. Only a few dehalogenases have been explored for their use in biocatalysis, even though their capacity to recognize and convert synthetic chemicals obviously can have great potential for industrial biotechnology. This review will focus on the dehalogenases that can be applied in the production of fine chemicals, in particular on the chlorocarboxylic acid dehalogenases, which have been investigated because of their stereoselectivity, the haloalkane dehalogenases, which so far have mainly bioremediation potential, and the haloalcohol dehalogenases, an emerging new class of highly interesting biocatalysts.

16.2 HALOALKANE DEHALOGENASES

16.2.1 PROPERTIES AND CATALYTIC MECHANISM

The biodegradation of chloro- and bromoalkanes often starts with a hydrolytic step in which the haloalkane is hydrolyzed to the corresponding alcohol. This occurs in the bacterial degradation of 1,2-dichloroethane, 1,3-dichloropropene, 1-chlorobutane, 1-chloro- and 1,6-dichlorohexane, chloroethylethers, and probably several other haloalkanes. All known haloalkane dehalogenases [16] are soluble enzymes of around 35 kDa and belong to the

α/β -hydrolase fold superfamily of enzymes, which also includes lipases, esterases, lactone hydrolases, carboxypeptidases, aminoester hydrolases, and epoxide hydrolases. The haloalkane dehalogenases are composed of two domains: the main domain, which has the conserved α/β -hydrolase fold topology, and a cap domain, which is comparable to the flexible lid of lipases.

Examples of well-studied haloalkane dehalogenases are Dh1A from *X. autotrophicus* [16,17], DhaA from *Rhodococcus erythropolis* [18], and LinB from *Sphingomonas paucimobilis* [19,20]. The former two enzymes act mainly on simple haloalkanes such as 1,2-dichloroethane, 1-chlorobutane, and 1,2-dibromoethane, whereas LinB converts 1,2,4,5-tetrachlorocyclohexadiene, an intermediate in the catabolic pathway for the insecticide γ -hexachlorocyclohexane (lindane). The active site in these enzymes is located in a cleft or cavity between the two domains. The main domain contributes three catalytic residues that are positioned in the form of a triad (acid/His/Asp), ordered along the sequence as Asp... acid... His. Structural and biochemical studies have revealed that the catalytic mechanism is based on the nucleophilicity of the conserved Asp residue, of which a carboxylate oxygen displaces the halogen from the substrate by nucleophilic substitution, forming a covalent intermediate (Figure 16.1). This mechanistic information was originally obtained from an x-ray structure in which the covalent intermediate was trapped by soaking the crystals of Dh1A with the substrate 1,2-dichloroethane at low pH [17]. The histidine, which is fully conserved in these dehalogenases, subsequently activates a water molecule that attacks the carbonyl carbon of the ester intermediate, releasing the alcohol product (Figure 16.1a). In other α/β -hydrolase fold enzymes, the nucleophilic Asp may be a Ser or Cys, like in esterases or lipases. The role of the acidic residue is to stabilize the positive charge developing on the histidine when the covalent intermediate is cleaved. It may be present at different topological positions and can be a Glu or an Asp. In Dh1A, it is an Asp, located in the sequence distal to the cap domain, whereas in DhaA and LinB it is a Glu located on a strand that precedes the cap [21–23].

An intriguing feature of the haloalkane dehalogenases is the presence of a distinct halide-binding site, which is essential for leaving group stabilization in the first half reaction where

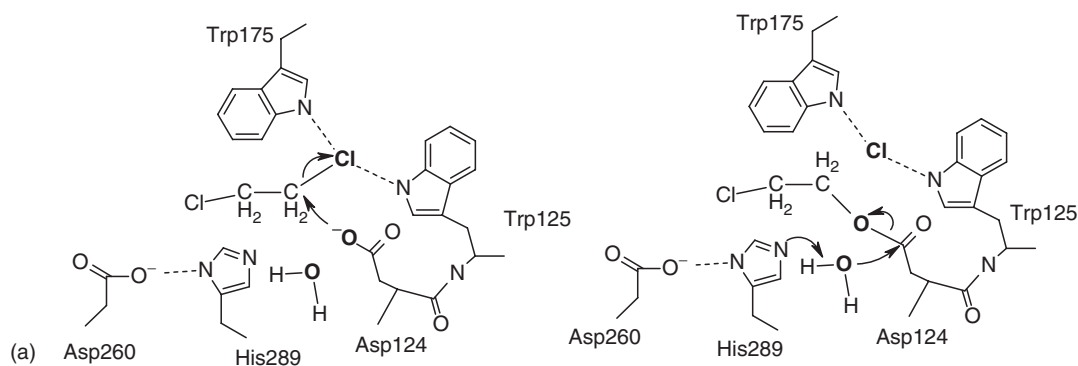


FIGURE 16.1 The catalytic mechanisms of three dehalogenases of different superfamilies. (a) Haloalkane dehalogenase (Dh1A) from *X. autotrophicus* GJ10. (From Ref. [17,80].)

(continued)

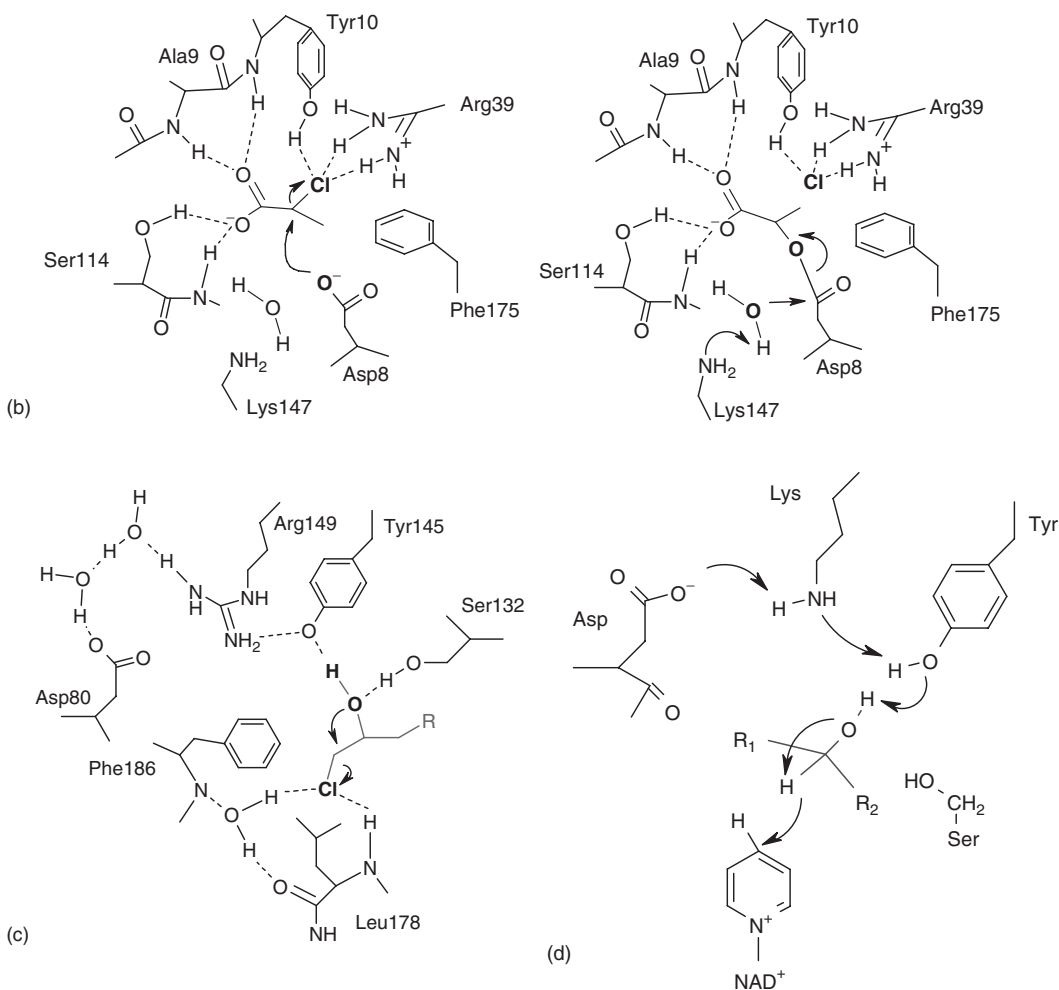


FIGURE 16.1 (continued) (b) Haloacid dehalogenase (HAD, DhlB) from *X. autotrophicus* GJ10. (From Ref. [47,48,51].) (c) Halohydrin dehalogenase (HheC) from *Agrobacterium radiobacter* AD1. (From Ref. [56,58,59].) (d) General mechanism of a short-chain dehydrogenase/reductase (SDR) enzyme.

the carbon–halogen bond is cleaved. The halide-binding site is composed of a Trp that flanks the nucleophilic Asp on the carboxy-terminal side and the side chain of a second residue, which is either contributed by the cap domain (Trp175 in DhlA) or by the main domain (Asn38 in LinB or Asn41 in DhaA). Sequence analysis has shown that catalytic residues are conserved in a much broader range of (putative) haloalkane dehalogenases [23]. In view of the conserved involvement in catalysis of five residues, the active site has been described as a catalytic pentad [16].

Several computational studies have confirmed that the catalytic mechanism that was revealed by x-ray crystallography for DhlA is energetically possible. They also provided further insight to the stabilizing interactions in the active site. For example, Bohac et al. [24]

concluded that electrostatic stabilization of the Trp residues is important for catalysis and emphasized the additional role of Phe172 in the stabilization of the halide in DhIA. This was in agreement with a kinetic analysis of Phe172 mutants [25]. Such mutants have a reduced rate of carbon–halogen bond cleavage, but still show an elevated activity (k_{cat}) for 1,2-dibromoethane conversion because halide release in the mutant has become faster, and this step, at the end of the catalytic cycle, is the slowest step during 1,2-dibromoethane hydrolysis in the wild-type enzyme [26,27]. More recent studies were aimed at describing the way in which haloalkane dehalogenase affects its remarkable catalytic power by comparing the reaction in the enzyme active site with that in water, and again confirmed the importance of electrostatic stabilization [28] and positioning of the desolvated reactant [29] close to the nucleophilic aspartate, but there is dispute about the most appropriate terminology and thermodynamic description of events that lead to transition-state stabilization [28].

16.2.2 ENVIRONMENTAL RELEVANCE

The use of free or immobilized enzymes instead of live cells in bioreactors for the cleanup of polluted water or soil does not seem to be attractive because of cost and limited enzyme stability under conditions that are difficult to control. Nevertheless, the general catalytic properties of dehalogenases have important environmental implications, since they will influence the spectrum of halogenated compounds that is degraded during natural attenuation of soil and groundwater pollutants as well as the possibility to develop treatment processes for the biological removal of halogenated compounds in reactors. Thus, the environmental recalcitrance of compounds such as 1,2-dichloropropane and 1,1,2-trichloroethane can be attributed to the fact that these organohalogens are hardly subject to microbial dehalogenation. A treatment process for the removal of 1,2-dichloroethane has been developed and implemented successfully owing to the availability of pure bacterial cultures that can rapidly degrade this compound and use it as a carbon source [30]. Thus, the discovery of new haloalkane dehalogenases with activity toward recalcitrant haloalkanes remains a topic of considerable importance, as will be discussed for 1,2,3-trichloropropane.

16.2.3 PROTEIN ENGINEERING STUDIES

Several attempts have been made to improve the catalytic activity of haloalkane dehalogenase. Initial work, carried out with DhIA, showed that mutations in the cap domain can enhance the activity for 1-chlorohexane [8]. The results showed that short tandem repeats varying in length from 3 to 10 amino acids caused a 5- to 25-fold increase in activity ($k_{\text{cat}}/K_{\text{m}}$) with 1-chlorohexane, whereas the specificity constant for 1,2-dichloroethane decreased up to 60-fold. It was found that the activity for 1,2-dibromoethane and other bromo compounds was much less influenced and the dehalogenation of bromoalkanes appears much less sensitive to mutations. Both with 1,2-dichloroethane and 1,2-dibromoethane, halide release is the slowest step in the catalytic cycle, which is accompanied by large conformational rearrangements in the cap domain [27]. For 1,2-dichloroethane, carbon–halogen bond cleavage is just a little bit faster than halide release. This causes loss of overall activity for 1,2-dichloroethane when halide release is made faster since the rates of carbon–halogen bond cleavage and halide release always appear to be inversely correlated. One can imagine that the former requires strong binding in the active site with precise positioning of the substrate, whereas the latter requires flexibility, which allows the active site to open and become solvated. A decrease of overall activity in mutants with a higher rate of halide release is not observed

for 1,2-dibromoethane, since carbon–bromine bond cleavage is very rapid anyway and remains fast enough in most mutants. Several mutations that modify the selectivity of DhIA have been described [25,31,32].

Damborsky et al. [33] have used computational methods to explore dehalogenase selectivity. Using a comparative binding energy analysis of dehalogenases with a series of ligands that was automatically docked into the active site, the position of several substrates was calculated. It appeared that potentially reactive binding modes for most substrates could be found. With 1,2-dibromopropane, two binding modes were uncovered, one allowing attack on the α carbon, and the other on the β -bromine-substituted carbon. This showed that hydrolysis can take place at either carbon atom, which is in agreement with experimental data (D.B. Janssen and J. Kingma, unpublished data).

These engineering and modeling studies also provide information about possible targets for further mutagenesis. Chaloupkova et al. [34] compared the three available haloalkane dehalogenase structures and concluded that residue Leu177 of LinB, located at the entrance of the tunnel leading to the active site and partly blocking it, might be a good target for mutagenesis. Mutants in which a smaller residue was present generally showed an enhanced catalytic activity. Apparently, substrate access and binding significantly influence the selectivity constant ($k_{\text{cat}}/K_{\text{m}}$) of this enzyme for most substrates.

It has been possible to obtain mutants of haloalkane dehalogenase with enhanced temperature stability. This also holds for DhIA, where a computational approach was used in which molecular dynamics simulation identified a flexible region in the dehalogenase protein that likely is an early unfolding region. It was subsequently fixed by introducing a disulfide bond, yielding a more resistant mutant [35]. The temperature stability of DhaA was significantly improved by a directed evolution approach [36].

In spite of all the computational and modeling work, no haloalkane dehalogenase variant has yet been obtained that attacks really difficult compounds such as 1,1,2-trichloroethane or 1,2-dichloropropane. Also no DhIA variants have yet been described that exhibit significantly better conversion of the “natural” substrate 1,2-dichloroethane [31]. Attempts to engineer or select a dehalogenase for one such remaining problematic compound are described below.

16.2.4 CONVERSION OF 1,2,3-TRICHLOROPROPANE

The industrial synthesis of epichlorohydrin is accompanied by significant formation of side products of which 1,2,3-trichloropropane (TCP) is the most important one. Biodegradation and biotransformation studies with this chemical have been aimed at obtaining a dehalogenase enzyme or a whole-cells system that can be used for the hydrolytic conversion of 1,2,3-trichloropropane. Researchers at Dow Chemical, working with Diversa, and we at the University of Groningen, working with Ciba Fine Chemicals, have explored the development of dehalogenases that can degrade 1,2,3-trichloropropane to 2,3-dichloro-1-propanol, starting with almost identical dehalogenases and using a directed evolution approach. After random mutagenesis, either by site-saturation mutagenesis or error-prone polymerase chain reaction (PCR), mutants with increased activity toward TCP have been obtained by Bosma et al. [37], which allowed slow growth of a recombinant bacterium on TCP. One of the two mutations was a Cys176Tyr substitution and, surprisingly, a mutation at this position also occurs in a natural variant of DhaA that we have obtained from a 1,2-dibromoethane degrading *Mycobacterium* strain [38]. Position Cys176 of DhaA corresponds to Leu177 in LinB, and its role in determining the selectivity of that enzyme has already been mentioned [34].

Gray et al. [39] have attempted to obtain a new collection of dehalogenases by exploring environmental gene libraries. An ingenious panning method based on sequence similarities was used to enrich a library for clones possessing haloalkane dehalogenase activity, and several new enzymes were indeed obtained. However, no dehalogenase variants have been isolated so far, either by directed evolution, genetic engineering, or the use of metagenome screening, that have sufficient activity to develop a feasible full-scale process for TCP hydrolysis at the level at which it is present in industrial waste.

16.2.5 ENANTIOSELECTIVITY

Little work has been done on the enantioselectivity of haloalkane dehalogenases, which would potentially be interesting for the preparation of enantiopure alkylhalides or alcohols by kinetic resolution. Pieters et al. [40] have demonstrated that haloalkane dehalogenases in principle can be enantioselective, but the enantioselectivity factors with the substrates that were tested were low (Figure 16.2, $E = 4$ to 9), both in kinetic resolutions and in the conversion of prochiral compounds such as 1,2,3-tribromopropane. Kinetic experiments showed that with methyl-3-bromo-methyl propionate, the large differences between the K_m values for the different enantiomers were compensated by large differences in k_{cat} that had the opposite effect. The enantioselectivity of the engineered DhaA haloalkane dehalogenase that showed enhanced 1,2,3-trichloropropane conversion was also low [37].

16.2.6 IMMOBILIZED HALOALKANE DEHALOGENASES

Bioreactors containing immobilized enzyme for treating water or gas that contains chloroalkanes as a contaminant have been described. Dravis et al. [41] studied conversion of 1-chlorobutane and 1,3-dichloropropane, supplied via the gas phase, by lyophilized DhaA

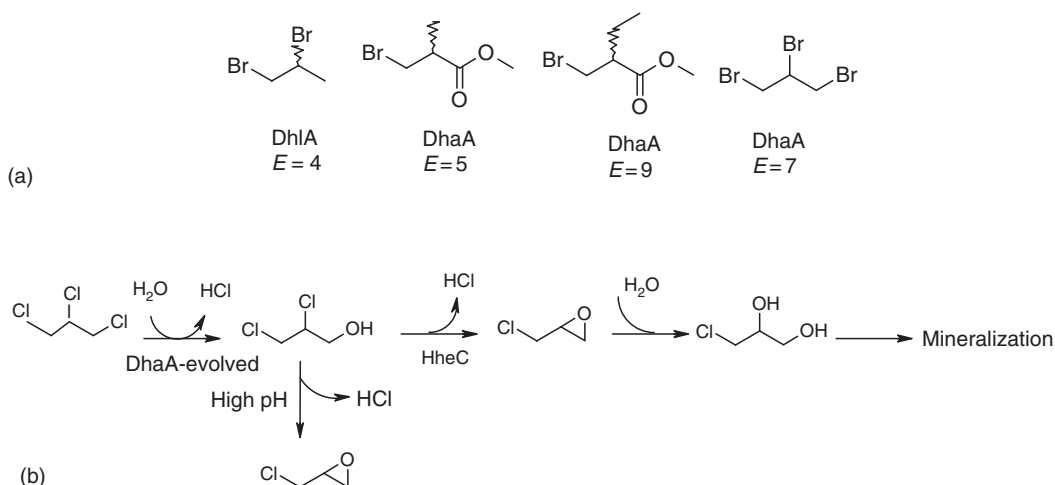


FIGURE 16.2 Biotechnologically relevant conversions catalyzed by haloalkane dehalogenases. (a) Substrates tested for kinetic resolution of haloalkane dehalogenases. (From Ref. [9].) E -values describe enantioselectivity [$E = (k_{cat,S}/K_{m,S})/(k_{cat,R}/K_{m,R})$]. (b) A potential pathway for trichloropropane mineralization or conversion to epichlorohydrin for reuse in manufacturing. (From Ref. [37,39,43].)

from *R. rhodochrous*. Although it seems counterintuitive to perform a reaction that requires water and produces hydrochloric acid in the gas phase, significant conversion was obtained. The specific activity for 1,3-dichloropropane was about 3.5×10^{-3} $\mu\text{mol}/\text{min}\cdot\text{mg}$ protein using enzyme lyophilized from high pH buffer at optimal water activity in the presence of triethylamine as a buffer. This activity is about 170-fold lower than the k_{cat} of the enzyme [42]. Immobilized haloalkane dehalogenase (DhaA) has also been tested for TCP removal after covalent immobilization on a polyethyleneimine impregnated alumina support. The immobilized enzyme was more stable than the free protein, whereas the thermostability and resistance to organic solvent were improved after covalent attachment of the enzyme to the support [43].

Other attempts to use haloalkane dehalogenase in the gas phase were published recently and involved the use of DhIA for 1,2-dichloroethane removal [44,45]. With lyophilized DhIA, the V_{max} was about 0.08 $\mu\text{mol}/\text{min}/\text{g}$ of cells, and it was found that activity and stability were dependent on the thermodynamic activity of the substrates and the water activity. As expected, the system displayed stability problems, which were attributed to accumulation of hydrochloric acid. If bioreactors must have a high space–time yield (or volumetric elimination rate), it is preferable to use packed bed systems with supply of buffer and removal of halide through the water phase. Trickle filters with immobilized whole cells seem to be a good option if rapid mass transfer of a compound that is sparingly soluble in water is required. They function well for dichloromethane removal from air [46], which is a more volatile organohalogen (in terms of partition coefficient) than 1,2-dichloroethane.

16.3 HALOACID DEHALOGENASES

16.3.1 PROPERTIES OF L-2-CHLOROPROPIONIC ACID DEHALOGENASES

A frequently studied group of aliphatic dehalogenases are the haloacid dehalogenases. Most of these enzymes can be classified as either group I or group II haloacid dehalogenases. The group II enzymes have been better characterized, and the x-ray structure of two of its members has been solved [47,48]. These proteins are somewhat smaller (about 29 kDa) than haloalkane dehalogenase, are active with the L-(S)-enantiomer of 2-chloropropionic acid, and convert this substrate with inversion of configuration at the chiral carbon atom. It is a diverse group of enzymes, members of which occur quite commonly, both in bacteria that grow on haloalkanoic acids and organisms that are not involved in haloalkanoic acid degradation. The structures confirmed the original proposal by Schneider et al. [49] that an aspartate residue located close to the N terminus of the protein acts as a nucleophile. Its carboxylate performs a nucleophilic displacement of the halogen, producing a covalent intermediate that is hydrolyzed by water in a similar way as the alkyl-enzyme intermediate in haloalkane dehalogenases (Figure 16.1b). The enzymes have a high pH optimum of around 9. The group II haloacid dehalogenases belong to the HAD superfamily of enzymes, which also includes proteins or protein domains that have phosphatase activity [50]. In these enzymes, the nucleophilic aspartate is also conserved.

The first haloacid dehalogenase whose structure was solved is the dimeric enzyme from *Pseudomonas* sp. YL was called L-DEX [47], and further information about the haloacid dehalogenase mechanism was obtained from structures in which the covalent intermediate was trapped by using an enzyme with a mutated oxyanion hole (Ser171Ala) [51] or by collecting data at low pH [48]. As in haloalkane dehalogenases, the active site is located between two domains. The haloacid dehalogenase main domain has an α/β structure, but the topology, in the sense of order and direction of strands in the β -sheet, is very different from that of haloalkane dehalogenases. In haloacid dehalogenase, the sheet of the main domain is composed of six parallel β -strands, and it is flanked by five α -helices. The second domain

is formed by an excursion in the sequence and has the topology of a four-helix bundle. The active site is lined by the nucleophilic Asp10 (in L-DEX), which is positioned on a loop that follows the N-terminal segment of the protein, and a series of other residues that influence the activity when mutated. Of the active site residues, Lys151 and Ser118 are also conserved in other haloacid dehalogenase proteins, such as phosphatases and an *N*-carbamoylsarcosine amidohydrolase from *Arthrobacter* sp. In the dehalogenases, the serine contributes to carboxylate binding, as do main chain amides of two amino acids that follow the nucleophilic aspartate. The conserved lysine could possibly act as the catalytic base that activates water for cleavage of the covalent intermediate. The halide-binding site in haloacid dehalogenases of the HAD superfamily is composed of an arginine (Arg41 in L-DEX, Arg39 in DhIA), and a tyrosine at a position +2 of the nucleophilic aspartate [48,51].

16.3.2 D-SPECIFIC CHLOROPROPIONIC ACID DEHALOGENASE

Another important group of haloacid dehalogenases are the D-specific 2-chloropropionic acid dehalogenases and phylogenetically related nonenantioselective dehalogenases, classified as group I enzymes [4]. Perhaps the best-characterized member of this group of proteins is a nonstereoselective haloacid dehalogenase from *Pseudomonas* sp. 113, called DL-DEX [52]. The protein has a single active site for converting both enantiomers of 2-chloropropionic acid. The sequence is similar to that of D-selective 2-haloacid dehalogenase from *Pseudomonas putida* AJ1, which only converts D-2-haloalkanoic acids. The catalytic mechanism was proposed to be fundamentally different from that of the HAD family of dehalogenases in the sense that the reaction does not involve a covalent intermediate. The conversion of 2-chloropropionic acid proceeds with inversion of configuration. Mechanistic details are not known, as no structure has been solved and sequence similarities to well-understood proteins are low.

16.3.3 PRODUCTION OF OPTICALLY ACTIVE CHLOROPROPIONIC ACID

The excellent enantioselectivity of some 2-chloropropionic acid dehalogenases and the need for optically active chloropropionic acid as a building block in the production of pharmaceuticals have triggered the development of a biocatalytic process for producing L-(*S*)-chloropropionic acid by kinetic resolution (Figure 16.3). This process has been commercialized in England by Zeneca (ICI) and the product is used for the manufacture of the phenoxyherbicide fusilade. After hydrolysis of the (*R*)-chloropropionic acid, the remaining (*S*)-chloropropionic acid is isolated by extraction and purified by distillation. The biocatalyst applied in this whole-cell process consisted of dried whole cells, which were stable enough for transport and storage [53].

Biotechnological processes for the production of enantiopure haloacids have been developed, for example, by Ordaz et al. [54] using immobilized L-(*S*)-chloropropionic dehalogenase, which was either His-tagged or coupled to an acrylic acid polymer. The L-2-haloacid dehalogenase DehCI from *Pseudomonas* CBS3 was used and the acrylate-immobilized enzyme was the more stable form. Immobilization of another 2-haloacid dehalogenase has also been done with a DEAE Sephacel solid matrix, which yielded an enzyme that was more temperature-stable and could be used in a plug-flow reactor [55].

16.4 HALOHYDRIN DEHALOGENASE

16.4.1 PROPERTIES

Halohydrin dehalogenases (also called haloalcohol hydrogen halide lyases) have been studied because of their environmental relevance and their potential application in biocatalysis. The

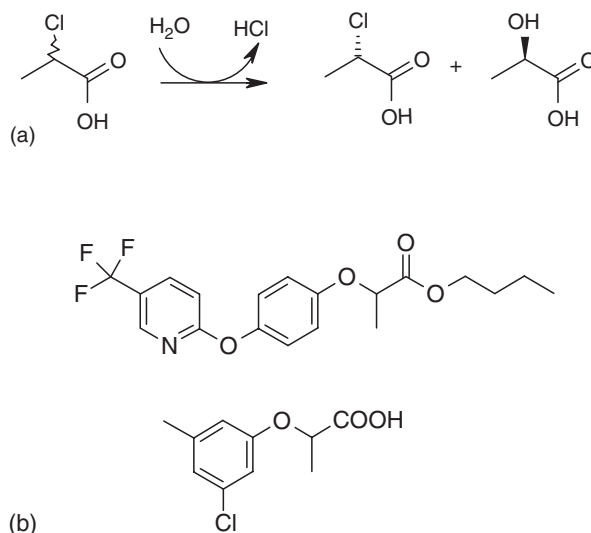


FIGURE 16.3 (a) Kinetic resolution of (*R,S*)-2-chloropropionic acid by a type I haloacid dehalogenase (HAD) to produce (b) (*S*)-2-chloropropionic acid, an intermediate for herbicides. (From Ref. [53].)

activity was first described by Castro and Bartnicki [3], who investigated the degradation of 2,3-dibromo-1-propanol. Later, halohydrin dehalogenases were purified and cloned from various organisms that degrade halohydrins [56,57], and the first x-ray structure was solved recently [58]. The latter work was done with HheC, a halohydrin dehalogenase from a strain of *Agrobacterium radiobacter* that grows on epichlorohydrin. The kinetics and substrate binding properties of this dehalogenase have been studied [59,60].

Sequence and structure analysis revealed that the catalytic mechanism of halohydrin dehalogenases is similar to that of members of the SDR superfamily of proteins. Both the dehalogenases and the SDR enzymes possess a conserved Ser/Tyr/Arg (or Ser/Tyr/Lys) catalytic triad for proton abstraction from the hydroxyl group of the substrate. In the case of SDR proteins, the negative charge developing on the hydroxyl oxygen is transferred by a hydride to the NAD(P)⁺ cofactor (Figure 16.1c). In the dehalogenases, instead, it is passed on with the oxygen to the neighboring carbon atom and the halogen bound to this carbon atom is displaced as halide. This intramolecular substitution mechanism results in the formation of an epoxide. The halohydrin dehalogenases thus use noncovalent catalysis. The enzymes also possess a distinct halide-binding site.

Sequence analysis of the known SDR-type halohydrin dehalogenases indicates that they can be divided into three different phylogenetic subgroups, which in this case coincides well with the classification according to substrate range. The group A enzymes (HheA from *Corynebacterium* sp. strain N-1074 and HheA_AD2 from *Agrobacterium*) have a high sequence similarity (97%). HheB from *Corynebacterium* sp. strain N-1074 and HheB-GP1 from *Mycobacterium* sp. GP1 are also very similar (98%). Only HheB is enantioselective in the conversion of 1,3-dichloropropanol to epichlorohydrin, producing the (*R*)-enantiomer [61]. In group C, the sequence similarity between HheC from *A. radiobacter* and HalB from *A. tumefaciens* is only 80%. HheC of *A. radiobacter* is the best-characterized halohydrin dehalogenase, both from a mechanistic and biocatalytic point of view, and distinguishes itself from most of the other enzymes because of its high enantioselectivity [62]. Another source of halohydrin dehalogenases is *Arthrobacter erithii* H10A [63].

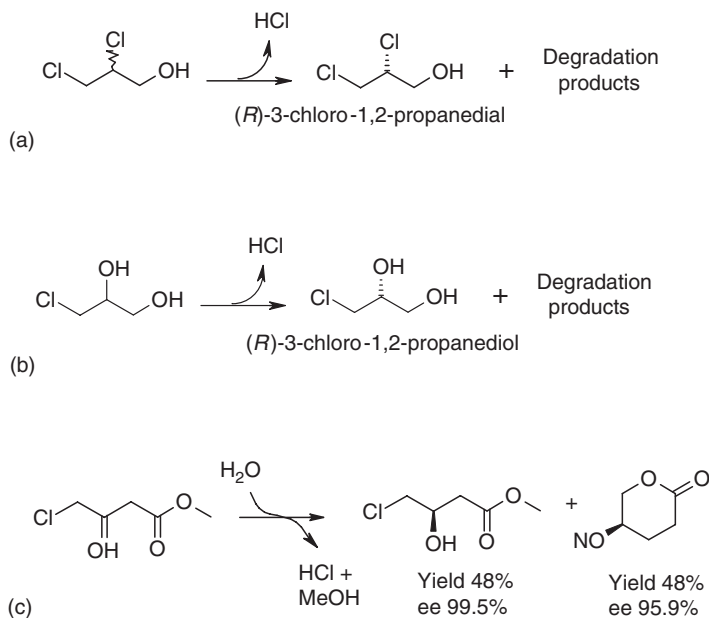


FIGURE 16.4 Dechlorination of chloropropanols and chlorinated C4 compounds catalyzed by halohydrin dehalogenases and whole cells containing dehalogenases. (a) Resolution of (*R,S*)-2,3-dichloro-1-propanol by enantioselective degradation with *Alcaligenes* sp. DS-K-S38. (From Ref. [64].) (b) Resolution of (*R,S*)-3-chloro-1,2-propanediol by enantioselective degradation with *Pseudomonas* sp. DS-K-2DI. (From Ref. [65].) (c) Enantioselective conversion of a 3-hydroxy-4-chlorobutyric acid methylester by an *Enterobacter* sp. (From Ref. [67].)

16.4.2 BIOCATALYTIC POTENTIAL OF HALOHYDRIN DEHALOGENATION

The enantioselectivity of halohydrin dehalogenases in the conversion of haloalcohols makes them promising biocatalysts for the production of optically active epoxides and β -substituted alcohols (Figure 16.4). Several bacterial cultures and enzymes that catalyze dechlorination of bromo- and chloropropanols have been investigated for their potential in the production of optically active C3 building blocks, and a historical account on the development of biocatalytic processes employing halohydrin dehalogenase containing microorganisms was published by Kasai et al. [64] and Kasai and Sizuki [65].

The epichlorohydrin precursor 2,3-dichloro-1-propanol has been produced in enantiomerically pure form by kinetic resolution using bacteria that possess halohydrin dehalogenase activity. In this process, the unwanted isomer is degraded. The (*S*)-enantiomer has been obtained using immobilized cells of *Pseudomonas* sp. OS-K-29, a strain that enantioselectively degrades the (*R*)-enantiomer. Organisms that selectively degrade (*S*)-2,3-dichloro-1-propanol were also found and allowed the production of (*R*)-2,3-dichloro-1-propanol. The later conversions have been performed at pilot scale, mostly using whole cells of *Alcaligenes* sp. DS-K-S38. The dehalogenase system in this organism is of a different type than the halohydrin dehalogenases of the SDR family class, and will be briefly discussed below. The optically active dichloropropanols that remain after the enantioselective degradation process can be isolated and used for the synthesis of a variety of products, including epichlorohydrin, which can be obtained from the halohydrins by simple chemical ring closure under basic conditions [64,65].

The use of halohydrin dehalogenase producing bacteria for making optically active 3-chloro-1,2-propanediols has also been studied with complementary organisms that have opposite stereospecificities. Kasai et al. [64] have screened several bacterial strains for the enantioselective conversion of these chiral chloropropanols. Although the enzymes involved are not described in detail, it is most likely that they are halohydrin dehalogenases related to the SDR family proteins. (*R*)-3-chloro-1,2-propanediol was obtained by kinetic resolution using whole cells of *Pseudomonas* sp. DS-K-2D1, whereas (*S*)-3-chloro-1,2-propanediol was obtained by enantioselective transformation with *Alcaligenes* sp. DS-S-7G [66].

Suzuki et al. [67] employed the stereoselectivity of halohydrin degradation system present in *Pseudomonas* sp. OS-K-29 to perform a kinetic resolution of 4-chloro-3-hydroxybutyrate ethylester. The remaining (*S*)-enantiomer had a high enantiomeric excess (ee) (>98%), although the yield was modest (33%), indicating that the enzyme involved did not have a very high enantioselectivity. The enzyme also appeared to be very tolerant with respect to the halohydrin R-group: 1-halo-2-hydroxybutane was converted, as well as the corresponding nitrile and different 4-halo-3-hydroxy-butanoic acid esters.

A conversion with the opposite enantioselectivity toward 4-chloro-3-hydroxybutyrate was also described [65]. In this case, the catalyst consisted of whole cells of *Pseudomonas* sp. DS-K-NR818 (a mutant of *Pseudomonas* sp. OS-K-29). When exposed to 4-chloro-3-hydroxybutyric acid ethyl ester, the (*S*)-enantiomer was degraded and the remaining ethyl (*R*)-4-chloro-3-hydroxybutyrate was recovered in good ee (>95%).

The conversions mentioned above showed the potential of biocatalytic production of enantiomerically pure halohydrins and related compounds with the use of whole cells. Using free enzyme, the halohydrin dehalogenase from *A. radiobacter* AD1 (HheC) was found to catalyze the dehalogenation of phenyl-substituted halohydrins with good enantioselectivity, yielding enantiomerically enriched (*R*)-epoxides and (*S*)-halohydrins [62]. The enantioselectivity was further increased by an active-site mutation (Trp249Phe), which also increased the k_{cat} for some substrates [68], whereas other mutations improved the stability against oxidation [69]. In the Trp249Phe mutant, the affected residue is donated by an opposite subunit and sticks in the binding site for the phenyl ring of styrene epoxide.

16.4.3 BIOCATALYTIC RING OPENING OF EPOXIDES

The possibility to use halohydrin dehalogenase for ring-opening reactions (Figure 16.5, Figure 16.6) is in line with the early observation that the enzymes catalyze transhalogenation [3]. An early alternative nucleophile to be explored was cyanide. Nakamura et al. [70] explored the epoxide ring opening of epoxybutyronitrile, a conversion that yielded (*R*)-4-chloro-3-hydroxybutyronitrile, an intermediate in the synthesis of L-carnithine. The enzyme was originated from a *Corynebacterium* sp.

The scope of epoxide ring-opening reactions was further explored by Lutje Spelberg et al. [71], using halohydrin dehalogenase from *A. radiobacter* (HheC). This enzyme catalyzes epoxide ring-opening reactions with several alternative nucleophiles, most notably with azide, cyanide, and nitrite, which allows the production of a range of β -substituted derivatives. The enantioselective azidolysis of styrene oxide and substituted derivatives yields 2-azido-1-phenylethanols of the (*R*) configuration, since the enzyme is enantioselective for (*R*)-epoxides in ring-opening reactions [72] (Figure 16.5). It was found that it is also possible to apply HheC in a dynamic kinetic resolution of epihalohydrins when using azide for ring opening [73]. This produces (*S*)-1-azidohaloalcohol. The racemization is based on the presence of catalytic amounts of halide, which opens the epoxide ring to produce nonchiral 1,3-dihalopropanol. With epichlorohydrin as the substrate, the rate of racemization was lower than the rate of ring opening by azide, resulting in a mixed kinetic resolution

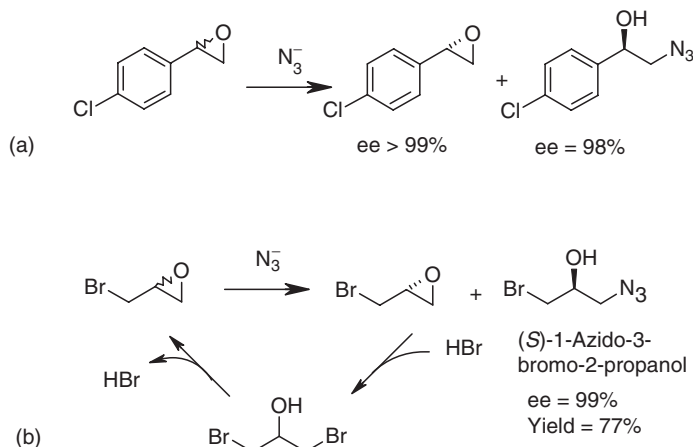


FIGURE 16.5 Epoxide ring-opening reaction catalyzed by HheC with azide as the nucleophile. (a) Enantioselective ring opening of *para*-chlorostyrene epoxide with azide. (From Ref. [72].) (b) Dynamic resolution of epibromohydrin. All reactions shown are catalyzed by HheC. (From Ref. [73].)

and dynamic kinetic resolution and modest enantiopurity of the (*S*)-1-azido-3-chloro-2-propanol. With epibromohydrin as the substrate, the racemization rate was higher, which allowed an efficient dynamic resolution and production of (*S*)-1-azido-3-bromo-2-propanol at $>99\%$ ee and with a yield of 77%. This product can be used for preparing additional chiral compounds.

Another unexpected reaction of halohydrin dehalogenases was the opening of epoxides with nitrite as the nucleophile [74]. Nitrite predominantly attacks the epoxide through its nitrite oxygen, yielding an unstable nitrite ester intermediate that spontaneously hydrolyzes to form the corresponding diol (Figure 16.7). This reaction occurs with high regioselectivity for the lesser substituted carbon atom of the oxirane ring and, depending on the epoxide substrate, with high enantioselectivity by using the W249F mutant halohydrin dehalogenase with improved enantiodiscrimination. This conversion makes it possible to apply halohydrin dehalogenases and nitrite as an alternative epoxide hydrolase to the kinetic resolution of various racemic epoxides. In several cases, the *E*-values are higher than those found with epoxide hydrolases, which catalyze the same overall reaction.

A further alternative nucleophile is cyanide (Figure 16.6). The HheC-mediated conversion of epoxides with this nucleophile was recently found to yield a variety of β -cyanoalcohols [75]. Again, the reactions proceed with varying enantioselectivity, depending on the epoxide. This conversion complements the reactions catalyzed by hydroxynitrile lyases. The latter enzymes mediate formation of α -hydroxynitriles from aldehydes, whereas halohydrin dehalogenases catalyze formation of β -hydroxynitriles from epoxides.

Another interesting application of halohydrin dehalogenases is in the manufacture of the statin side-chain building block 4-cyano-3-hydroxybutyric acid ester (Figure 16.6). Starting from 4-bromo-3-ketobutyric acid ethylester, this product was obtained by a glucose dehydrogenase-mediated reduction to (3*S*)-hydroxybutyric acid, which was subsequently converted by the epoxide to the product by an engineered HheC-type halohydrin dehalogenase [76]. This is one of the several possible routes to the statin side chain [77].

In conclusion, halohydrin dehalogenases are an important emerging new class of biocatalysts. They show unprecedented catalytic promiscuity by being able to catalyze carbon–chlorine, carbon–bromine, carbon–nitrogen, carbon–oxygen, and carbon–carbon bond formation. I hypothesize

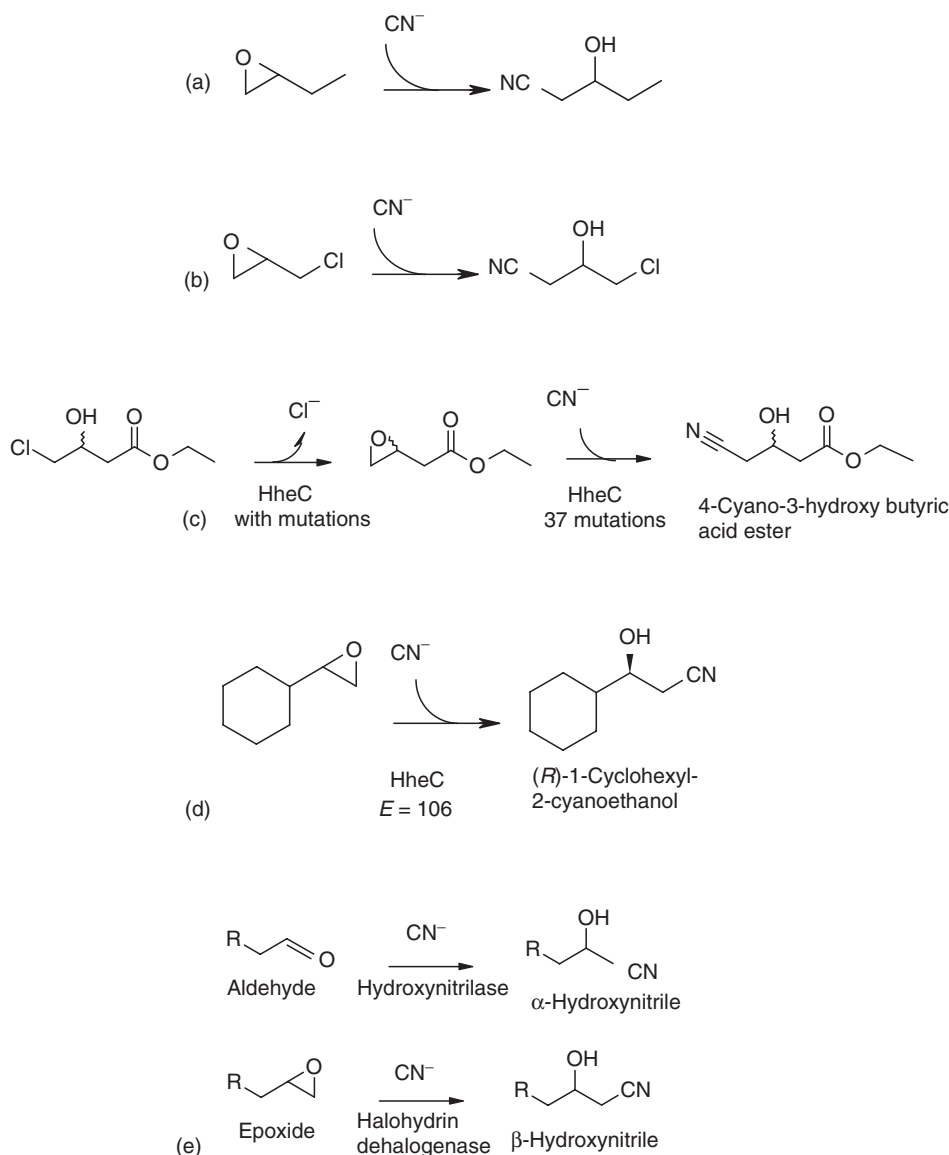


FIGURE 16.6 Epoxide ring opening with cyanide as catalyzed by halohydrin dehalogenases. (a) Ring opening of epoxybutane to produce hydroxyvaleronitrile. (From Ref. [70].) (b) Ring opening of epichlorohydrin to produce (*R*)-4-chloro-3-hydroxybutyronitrile. (From Ref. [81].) (c) Conversion of a 4-halo-3-hydroxybutyric acid ester to the corresponding nitrile for producing a statin side-chain building block. (From Ref. [76].) (d) Enantioselective conversion of cyclohexyloxirane to the corresponding cyanohydrin. (From Ref. [75].) (e) Comparison of halohydrin dehalogenase and hydroxynitrilase-mediated reactions.

that halohydrin dehalogenases are evolutionary primitive enzymes that have not evolved a high selectivity toward a specific substrate. The nonspecific active site can be used for a wide diversity of reactions. It is well possible that bacterial genomes harbor many such genes, which encode promiscuous enzymes that can be tailored to catalyze industrially important reactions.

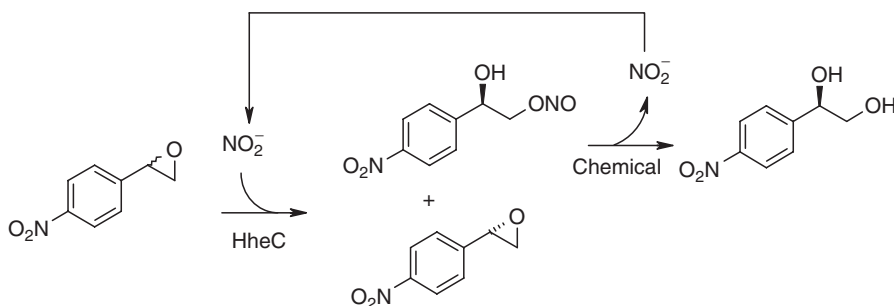


FIGURE 16.7 Nitrite-mediated ring opening of *para*-nitrostyrene epoxide catalyzed by HheC. The (*R*)-enantiomer is converted to a nitrite ester. This unstable product is hydrolyzed to the diol, with recovery of nitrite. The net reaction is an epoxide hydrolase-like kinetic resolution. (From Ref. [74].)

16.4.4 OTHER DEHALOGENASES THAT CONVERT HALOHYDRINS

An interesting intramolecular transesterification that yields dehalogenation has been described by Suzuki et al. [78] although its biochemistry has not yet been studied well. A culture of *Enterobacter* sp. strain DS-S-75 could stereoselectively dechlorinate in a racemic mixture the (*S*)-enantiomer of (*S*)-4-chloro-3-hydroxybutyrate ethylester to yield the remaining (*R*)-4-chloro-3-hydroxybutyrate ester with excellent optical purity. Interestingly, the converted (*S*)-enantiomer was not just dechlorinated but also transformed into (*S*)-3-hydroxy- γ -butyrolactone, releasing the corresponding alcohol moiety of the substrate and chloride. This activity required a carboxylate and a halogen group at terminal positions.

As mentioned earlier, one of the bacteria known to degrade 2,3-dichloro-1-propanol is *Alcaligenes* sp. DS-S-7G [79]. The responsible two-component enzyme from this organism has a very broad substrate range and is enantioselective for the (*R*)-enantiomer. The dehalogenation mechanism is complicated and involves a 70 kDa protein that contains flavin adenine dinucleotide (FAD) and oxidizes the substrate in an NAD-dependent reaction. The second component is composed of 33 kDa and 53 kDa subunits and seems to be involved in transfer or regeneration of the electron transfer components. It was proposed that the reaction proceeds through 3-chloroacetone, which would decompose chemically in a reaction in which the halogen is replaced by a hydrogen. Details of this mechanism and the exact stoichiometry and role of the protein components and reactants have not been worked out. This is another illustration of the fact that several unusual enzyme systems for halohydrin dehalogenation still await exploration at the biochemical level.

16.5 CONCLUSIONS AND OUTLOOK

The current status of the research on dehalogenases has yielded fascinating insight into the molecular mechanism of biological carbon-halogen bond cleavage and the diversity of enzymes that can catalyze for such reactions. Development of whole-cell processes in which dehalogenase-producing organisms are applied for removing chlorinated compounds in bioremediation schemes has been successful. Various enzyme systems for dehalogenation reactions have hardly been explored, and it is likely that surprising new enzyme mechanisms can still be found. We also foresee a growth in the range of reactions that are useful for biocatalysis, especially when new enzymes that attack a broader range of organic substrates are found.

REFERENCES

1. Davies, J.I. and Evans, W.C., The elimination of halide ions from aliphatic halogen-substituted organic acids by an enzyme preparation from *Pseudomonas dehalogenans*, *Biochem. J.*, 82, 50–51, 1962.
2. Goldman, P., The enzymatic cleavage of the carbon–fluorine bond in fluoroacetate, *J. Biol. Chem.*, 240, 3434–3438, 1965.
3. Castro, C.E. and Bartnicki, E.W., Biodehalogenation, epoxidation of halohydrins, epoxide opening, and transhalogenation by a *Flavobacterium* sp., *Biochemistry*, 7, 3213–3218, 1968.
4. Hill, K.E., Marchesi, J.R., and Weightman, A.J., Investigation of two evolutionarily unrelated halocarboxylic acid dehalogenase gene families, *J. Bacteriol.*, 181, 2535–2547, 1999.
5. Nardi-Dei, V., Kurihara, T., Park, C., et al., DL-2-Haloacid dehalogenase from *Pseudomonas* sp. 113 is a new class of dehalogenase catalyzing hydrolytic dehalogenation not involving enzyme–substrate ester intermediate, *J. Biol. Chem.*, 274, 20977–20981, 1999.
6. Smidt, H., Akkermans, A.D.L., van der Oost, J., and de Vos, W.M., Halorespiring bacteria—molecular characterization and detection, *Enzyme Microb. Technol.*, 27, 812–820, 2000.
7. Gribble, G.W., Naturally occurring organohalogen compounds, *Acc. Chem. Res.*, 31, 141–152, 1998.
8. Pries, F., van den Wijngaard, A.J., Bos, R., et al., The role of spontaneous cap domain mutations in haloalkane dehalogenase specificity and evolution, *J. Biol. Chem.*, 269, 17490–17494, 1994.
9. Pikkemaat, M.G. and Janssen, D.B., Generating segmental mutations in haloalkane dehalogenase: a novel part in the directed evolution toolbox, *Nucleic Acids Res.*, 30, E35–5, 2002.
10. Seffernick, J.L., de Souza, M.L., Sadowsky, M.J., and Wackett, L.P., Melamine deaminase and atrazine chlorohydrolase: 98 percent identical but functionally different, *J. Bacteriol.*, 183, 2405–2410, 2001.
11. Wackett, L.P., Evolution of enzymes for the metabolism of new chemical inputs into the environment, *J. Biol. Chem.*, 279, 41259–41262, 2004.
12. Poelarends, G.J. and Whitman, C.P., Evolution of enzymatic activity in the tautomerase superfamily: mechanistic and structural studies of the 1,3-dichloropropene catabolic enzymes, *Bioorg. Chem.*, 32, 376–392, 2004.
13. Poelarends, G.J., Saunier, R., and Janssen, D.B., *trans*-3-chloroacrylic acid dehalogenase from *Pseudomonas pavonaceae* 170 shares structural and mechanistic similarities with 4-oxalocrotonate tautomerase, *J. Bacteriol.*, 183, 4269–4277, 2001.
14. Horvat, C.M. and Wolfenden, R.V., A persistent pesticide residue and the unusual catalytic proficiency of a dehalogenating enzyme, *Proc. Natl. Acad. Sci. USA*, 102, 16199–16202, 2005.
15. de Jong, R.M., Brugman, W., Poelarends, G.J., et al., The x-ray structure of *trans*-3-chloroacrylic acid dehalogenase reveals a novel hydration mechanism in the tautomerase superfamily, *J. Biol. Chem.*, 279, 11546–11552, 2004.
16. Janssen, D.B., Evolving haloalkane dehalogenases, *Curr. Opin. Chem. Biol.*, 8, 150–159, 2004.
17. Verschuere, K.H., Seljee, F., Rozeboom, H.J., et al., Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase, *Nature*, 363, 693–698, 1993.
18. Newman, J., Peat, T.S., Richard, R., et al., Haloalkane dehalogenases: structure of a *Rhodococcus* enzyme, *Biochemistry*, 38, 16105–16114, 1999.
19. Marek, J., Vevodova, J., Smatanova, I.K., et al., Crystal structure of the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26, *Biochemistry*, 39, 14082–14086, 2000.
20. Prokop, Z., Monincova, M., Chaloupkova, R., et al., Catalytic mechanism of the haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* UT26, *J. Biol. Chem.*, 278, 45094–45100, 2003.
21. Krooshof, G.H., Ridder, I.S., Tepper, A.W., et al., Kinetic analysis and x-ray structure of haloalkane dehalogenase with a modified halide-binding site, *Biochemistry*, 37, 15013–15023, 1998.
22. Hynkova, K., Nagata, Y., Takagi, M., and Damborsky, J., Identification of the catalytic triad in the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26, *FEBS Lett*, 446, 177–181, 1999.
23. Damborsky, J. and Koca, J., Analysis of the reaction mechanism and substrate specificity of haloalkane dehalogenases by sequential and structural comparisons, *Protein Eng.*, 12, 989–998, 1999.

24. Bohac, M., Nagata, Y., Prokop, Z., et al., Halide-stabilizing residues of haloalkane dehalogenases studied by quantum mechanic calculations and site-directed mutagenesis, *Biochemistry*, 41, 14272–14280, 2002.
25. Schanstra, J.P., Ridder, I.S., Heimeriks, G.J., et al., Kinetic characterization and x-ray structure of a mutant of haloalkane dehalogenase with higher catalytic activity and modified substrate range, *Biochemistry*, 35, 13186–13195, 1996.
26. Schanstra, J.P., Kingma, J., and Janssen, D.B., Specificity and kinetics of haloalkane dehalogenase, *J. Biol. Chem.*, 271, 14747–14753, 1996.
27. Schanstra, J.P. and Janssen, D.B., Kinetics of halide release of haloalkane dehalogenase: evidence for a slow conformational change, *Biochemistry*, 35, 5624–5632, 1996.
28. Shurki, A., Strajbl, M., Villa, J., and Warshel, A., How much do enzymes really gain by restraining their reacting fragments? *J. Am. Chem. Soc.*, 124, 4097–4107, 2002.
29. Lau, E.Y., Kahn, K., Bash, P.A., and Bruice, T.C., The importance of reactant positioning in enzyme catalysis: a hybrid quantum mechanics/molecular mechanics study of a haloalkane dehalogenase, *Proc. Natl. Acad. Sci. USA*, 97, 9937–9942, 2000.
30. Stucki, G. and Thüer, M., Experiences of a large-scale application of 1,2-dichloroethane degrading microorganisms for groundwater treatment, *Environ. Sci. Technol.*, 29, 2339–2345, 1995.
31. Schanstra, J.P., Ridder, A., Kingma, J., and Janssen, D.B., Influence of mutations of Val226 on the catalytic rate of haloalkane dehalogenase, *Protein Eng.*, 10, 53–61, 1997.
32. Holloway, P., Knoke, K.L., Trevors, J.T., and Lee H., Alteration of the substrate range of haloalkane dehalogenase by site-directed mutagenesis, *Biotechnol. Bioeng.*, 59, 520–523, 1998.
33. Kmunicek, J., Bohac, M., Luengo, S., et al., Comparative binding energy analysis of haloalkane dehalogenase substrates: modelling of enzyme–substrate complexes by molecular docking and quantum mechanical calculations, *J. Comput. Aided Mol. Des.*, 17, 299–311, 2003.
34. Chaloupkova, R., Sykorova, J., Prokop, Z., et al., Modification of activity and specificity of haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26 by engineering of its entrance tunnel, *J. Biol. Chem.*, 278, 52622–52628, 2003.
35. Pikkemaat, M.G., Linssen, A.B., Berendsen, H.J., and Janssen, D.B., Molecular dynamics simulations as a tool for improving protein stability, *Protein Eng.*, 15, 185–192, 2002.
36. Gray, K.A., Richardson, T.H., Kretz K., et al., Rapid evolution of reversible denaturation and elevated melting temperature in a microbial haloalkane dehalogenase, *Adv. Synth. Catal.*, 343, 607–617, 2001.
37. Bosma, T., Damborsky, J., Stucki, G., and Janssen, D.B., Biodegradation of 1,2,3-trichloropropane through directed evolution and heterologous expression of a haloalkane dehalogenase gene, *Appl. Environ. Microbiol.*, 68, 3582–3587, 2002.
38. Poelarends, G.J., van Hylckama Vlieg, J.E.T., Marchesi, J.R., et al., Degradation of 1,2-dibromoethane by *Mycobacterium* sp. strain GP1, *J. Bacteriol.*, 181, 2050–2058, 1999.
39. Gray, K.A., Richardson, T.H., Robertson, D.E., et al., Soil-based gene discovery: a new technology to accelerate and broaden biocatalytic applications, *Adv. Appl. Microbiol.*, 52, 1–27, 2003.
40. Pieters, R.J., Fennema, M., Kellogg, R.M., and Janssen, D.B., Design and synthesis of reagents for phage display screening of dehalogenases, *Bioorg. Med. Chem. Lett.*, 9, 161–166, 1999.
41. Dravis, B.C., LeJeune, K.E., Hetro, A.D., and Russell, A.J., Enzymatic dehalogenation of gas phase substrates with haloalkane dehalogenase, *Biotechnol. Bioeng.*, 69, 235–241, 2000.
42. Bosma, T., Pikkemaat, M.G., Kingma, J., et al., Steady-state and pre-steady-state kinetic analysis of halopropane conversion by a *Rhodococcus* haloalkane dehalogenase, *Biochemistry*, 42, 8047–8053, 2003.
43. Dravis, B.C., Swanson, P.E., and Russell, A.J., Haloalkane hydrolysis with an immobilized haloalkane dehalogenase, *Biotechnol. Bioeng.*, 75, 416–423, 2001.
44. Erable, B., Goubet I., Lamare S., et al., Haloalkane hydrolysis by *Rhodococcus erythropolis* cells: comparison of conventional aqueous phase dehalogenation and nonconventional gas phase dehalogenation, *Biotechnol. Bioeng.*, 86, 47–54, 2004.
45. Erable, B., Goubet, I., Lamare, S., Seltana, A., et al., Nonconventional hydrolytic dehalogenation of 1-chlorobutane by dehydrated bacteria in a continuous solid–gas biofilter, *Biotechnol. Bioeng.*, 9, 304–313, 2005.

46. Diks, R.M.M. and Ottengraf, S.P.P., Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter: part II, *Bioproc. Eng.*, 6, 131–140, 1991.
47. Hisano, T., Hata, Y., Fujii, T., et al., Crystal structure of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL. An alpha/beta hydrolase structure that is different from the alpha/beta hydrolase fold, *J. Biol. Chem.*, 271, 20322–20330, 1996.
48. Ridder, I.S., Rozeboom, H.J., Kalk, K.H., and Dijkstra, B.W., Crystal structures of intermediates in the dehalogenation of haloalkanoates by L-2-haloacid dehalogenase, *J. Biol. Chem.*, 274, 30672–30678, 1999.
49. Schneider, B., Muller, R., Frank, R., and Lingens, F., Site-directed mutagenesis of the 2-haloalkanoic acid dehalogenase I gene from *Pseudomonas* sp. strain CBS3 and its effect on catalytic activity, *Biol. Chem. Hoppe Seyler*, 374, 489–496, 1993.
50. Aravind, L., Galperin, M.Y., and Koonin, E.V., The catalytic domain of the P-type ATPase has the haloacid dehalogenase fold, *Trends Biochem. Sci.*, 23, 127–129, 1998.
51. Li, Y.F., Hata, Y., Fujii, T., et al., Crystal structures of reaction intermediates of L-2-haloacid dehalogenase and implications for the reaction mechanism, *J. Biol. Chem.*, 273, 15035–15044, 1998.
52. Nardi-Dei, V., Kurihara, T., Park, C., et al., DL-2-Haloacid dehalogenase from *Pseudomonas* sp. 113 is a new class of dehalogenase catalyzing hydrolytic dehalogenation not involving enzyme–substrate ester intermediate, *J. Biol. Chem.*, 274, 20977–20981, 1999.
53. OECD, The application of biotechnology to industrial sustainability: manufacture of (S)-chloropropionic acid (Avecia, United Kingdom), OECD, Paris, 2001, pp. 67–90.
54. Ordaz, E., Garrido-Pertierra, A., Gallego, M., and Puyet, A., Covalent and metal–chelate immobilization of a modified 2-haloacid dehalogenase for the enzymatic resolution of optically active chloropropionic acid, *Biotechnol. Prog.*, 16, 287–291, 2000.
55. Diez, A., Prieto, M.I., Alvarez, M.J., et al., Improved catalytic performance of a 2-haloacid dehalogenase from *Azotobacter* sp. by ion-exchange immobilisation, *Biochem. Biophys. Res. Commun.*, 220, 828–833, 1996.
56. van Hylckama Vlieg, J.E.T., Tang, L., Lutje Spelberg, J.H., et al., Halohydrin dehalogenases are structurally and mechanistically related to short-chain dehydrogenases/reductases, *J. Bacteriol.*, 183, 5058–5066, 2001.
57. Yu, F., Nakamura, T., Mizunashi, W., and Watanabe, I., Cloning of two halohydrin hydrogen-halide-lyase genes of *Corynebacterium* sp. strain N-1074 and structural comparison of the genes and gene products, *Biosci. Biotechnol. Biochem.*, 58, 1451–1457, 1994.
58. de Jong, R.M., Tiesinga, J.J., Rozeboom, H.J., et al., Structure and mechanism of a bacterial haloalcohol dehalogenase: a new variation of the short-chain dehydrogenase/reductase fold without an NAD(P)H binding site, *EMBO J.*, 22, 4933–4944, 2003.
59. Tang, L., Lutje Spelberg, J.H., Fraaije, M.W., and Janssen, D.B., Kinetic mechanism and enantioselectivity of halohydrin dehalogenase from *Agrobacterium radiobacter*, *Biochemistry*, 42, 5378–5386, 2003.
60. Tang, L., van Merode, A.E., Lutje Spelberg, J.H., et al., Steady-state kinetics and tryptophan fluorescence properties of halohydrin dehalogenase from *Agrobacterium radiobacter*: roles of W139 and W249 in the active site and halide-induced conformational change, *Biochemistry*, 42, 14057–14065, 2003.
61. Nakamura, T., Nagasawa, T., Yu, F., et al., Resolution and some properties of enzymes involved in enantioselective transformation of 1,3-dichloro-2-propanol to (R)-3-chloro-1,2-propanediol by *Corynebacterium* sp. strain N-1074, *J. Bacteriol.*, 174, 7613–7619, 1992.
62. Lutje Spelberg, J.H.L., van Hylckama Vlieg, J.E.T., Bosma, T., et al., A tandem enzyme reaction to produce optically active halohydrins, epoxides and diols, *Tetrahedron Asymmetry*, 15, 2863–2870, 1999.
63. Assis, H.M., Sallis, P.J., Bull, A.T., and Hardman, D.J., Biochemical characterization of a haloalcohol dehalogenase from *Arthrobacter erithii* H10a, *Enzyme Microb. Technol.*, 22, 568–574, 1998.
64. Kasai, N., Suzuki, S., and Furukawa, Y., Optically active chlorohydrins as chiral C3 and C4 building units: microbial resolution and synthetic applications, *Chirality*, 10, 682–692, 1998.
65. Kasai, N. and Suzuki, T., Industrialization of the microbial resolution of chiral C3 and C4 synthetic units: from a small beginning to a major operation, a personal account, *Adv. Synth. Catal.*, 345, 437–455, 2003.

66. Suzuki, T. and Kasai, N., A novel method for the generation of (*R*)- and (*S*)-3-chloro-1,2-propanediol by stereospecific dehalogenating bacteria and their use in the preparation of (*R*)- and (*S*)-glycidol, *Bioorg. Med. Chem. Lett.*, 1, 343–346, 1991.
67. Suzuki, T., Idogaki, H., and Kasai, N., A novel generation of optically active ethyl-4-chloro-3-hydroxybutyrate as a C4 chiral building unit using microbial dechlorination, *Tetrahedron Asymmetry*, 7, 3109–3112, 1996.
68. Tang, L., Torres Pazmino, D.E., Fraaije, M.W., et al., Improved catalytic properties of halohydrin dehalogenase by modification of the halide-binding site, *Biochemistry*, 44, 6609–6618, 2005.
69. Tang, L., van Hylckama Vlieg, J.E.T., Fraaije, M., and Janssen, D.B., Improved stability of halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 by replacement of cysteine residues, *Enzyme Microb. Technol.*, 30, 251–258, 2002.
70. Nakamura, T., Nagasawa, T., Yu, F., et al., A new catalytic function of halohydrin hydrogen-halide-lyase, synthesis of beta-hydroxynitriles from epoxides and cyanide, *Biochem. Biophys. Res. Commun.*, 180, 124–130, 1991.
71. Lutje Spelberg, J.H., Tang, L., van Gelder, M., et al., Exploration of the biocatalytic potential of a halohydrin dehalogenase using chromogenic substrates, *Tetrahedron Asymmetry*, 13, 1083–1089, 2002.
72. Lutje Spelberg, J.H., van Hylckama Vlieg, J.E., Tang, L., et al., Highly enantioselective and regioselective biocatalytic azidolysis of aromatic epoxides, *Org. Lett.*, 3, 41–43, 2001.
73. Lutje Spelberg, J.H., Tang, L., Kellogg, R.M., and Janssen, D.B., Enzymatic dynamic kinetic resolution of epihalohydrins, *Tetrahedron Asymmetry*, 15, 1095–1102, 2004.
74. Hasnaoui, G., Lutje Spelberg, J.H., de Vries, E., et al., Nitrite-mediated hydrolysis of epoxides catalyzed by halohydrin dehalogenase from *Agrobacterium radiobacter* AD1: a new tool for the kinetic resolution of epoxides, *Tetrahedron Asymmetry*, 16, 1685–1692, 2005.
75. Majerić-Elenkov, M., Hauer, B., and Janssen, D.B., Enantioselective ring opening of epoxides with cyanide catalysed by halohydrin dehalogenases: a new approach to enantiopure β -hydroxynitriles, *Adv. Synth. Catal.*, 348, 579–585, 2006.
76. Davis, S.C., Grate, J.H., Gray, D.R., et al., Enzymatic processes for the production of 4-substituted 3-hydroxybutyric acid derivatives and vicinal cyano, hydroxy substituted carboxylic acid esters, Patent WO 2004/015132.
77. Müller, M., Chemoenzymatic synthesis of building blocks for statin side chains, *Angew. Chem. Intl. Ed.*, 44, 362–365, 1995.
78. Suzuki, T., Idogaki, H., and Kasai, N., Dual production of highly pure methyl (*R*)-4-chloro-3-hydroxybutyrate and (*S*)-3-hydroxy- γ -butyrolactone with *Enterobacter* sp., *Enzyme Microb. Technol.*, 24, 13–20, 1999.
79. Suzuki, T., Kasai, N., Yamamoto, R., and Minamiura, N., A novel enzymatic dehalogenation of (*R*)-3-chloro-1,2-propanediol in *Alcaligenes* sp. DS-S-7G, *Appl. Microbiol. Biotechnol.*, 42, 270–279, 1994.
80. Pries, F., Kingma, J., Pentenga, M., et al., Site-directed mutagenesis and oxygen isotope incorporation studies of the nucleophilic aspartate of haloalkane dehalogenase, *Biochemistry*, 33, 1242–1247, 1994.
81. Nakamura, T., Nagasawa, T., Yu, F., et al., A new enzymatic synthesis of (*R*)- γ -chloro- β -hydroxybutyronitrile, *Tetrahedron*, 50, 11821–11826, 1994.

17 Enzymatic Synthesis of Sugar Esters and Oligosaccharides from Renewable Resources

*A. Ballesteros, F.J. Plou, M. Alcalde, M. Ferrer,
H. García-Arellano, D. Reyes-Duarte, and I. Ghazi*

CONTENTS

17.1	Carbohydrates and Fats as Renewable Feedstock.....	463
17.2	Synthesis of Fatty Acid Sugar Esters by Lipases and Proteases	464
17.2.1	Enzyme Regiospecificity.....	465
17.2.2	Reaction Medium	466
17.2.3	Acyl Donors.....	469
17.3	Synthesis of Oligosaccharides by Transglycosidases	470
17.3.1	Transfructosidases.....	473
17.3.1.1	Synthesis of Inulin-Type Fructooligosaccharides.....	474
17.3.1.2	Synthesis of ⁶ F-Type and Neo-Fructooligosaccharides.....	475
17.3.2	Transglucosidases.....	476
17.3.2.1	Glucansucrases	476
17.3.2.2	Cyclodextrin Glucosyltransferases	478
	Acknowledgments	481
	References	481

17.1 CARBOHYDRATES AND FATS AS RENEWABLE FEEDSTOCK

As fossil raw materials are diminishing, and as the pressure on our environment is strengthening, the progressive switch of industry toward renewable feedstock emerges as an unavoidable necessity [1]. In fact, one of the basic principles of Green Chemistry is to develop processes that use renewable starting materials as an alternative to fossil resources [2]. Plant biomass represents the dominant source of feedstock for biotechnological processes as well as the only predictable sustainable source of organic fuels, chemicals, and materials [3]. For the best utilization of biomass, it first needs to be separated into its principal components: cellulose, hemicellulose, lignin, xylooligomers, starch, nonstructural carbohydrates, vegetable and essential oils, etc.

Carbohydrates are considered, by far, the most important class of renewable compounds. About 200 billion MT/y of glucose are formed by photosynthesis, of which 95% remains in the carbohydrate stage as mono-, oligo-, and polysaccharides and glycoconjugates, whereas the remaining 5% is transformed through metabolic pathways into amino acids and later into proteins, nucleic acids, and steroids [4]. A minor fraction (~4%) of the total carbohydrates

produced by nature is used by man [1], and the rest decays and recycles along natural pathways [5].

Only a few carbohydrates fulfill the criteria of low price, quality, reactivity, and availability to constitute interesting raw material sources. Polysaccharides constitute the bulk of the renewable carbohydrate biomass (Table 17.1). Nowadays, corn is the dominant feedstock for biological productions, as its carbohydrate (starch) is in a form that is more homogeneous and more reactive than that found in cellulosic materials [3]. The disaccharide sucrose—from sugar beet or sugar cane—is the most abundant pure organic molecule produced at the industrial scale ($>125 \times 10^6$ MT/y) [6]. Finally, the constituent repeating units of the main polysaccharides—glucose (cellulose, starch), fructose (inulin), and xylose (xylan)—are inexpensive and available on multiton scale. Thus, D-glucose is produced from starch in the form of syrups or pure crystals [1]. Fructose is prepared by isomerization of starch-derived glucose or by hydrolysis of inulin. Sorbitol, the hydrogenated glucose derivative, is also widely available [7].

Together with carbohydrates, plant oils and animal fats play an important role as renewable resources because of their availability and versatile applications [5,8]. While the largest share ($\sim 100 \times 10^6$ MT/y) of fats and oils is used as human foodstuff, 15% is available for oleochemistry (soaps, detergents, cosmetics, biodiesel, lubricants, etc.) [5].

In general, carbohydrates and plant oils are attractive renewable raw materials for the production of a wide range of food and chemical products, including pharmaceuticals, agrochemicals, insecticides, and novel biodegradable surfactants [9]. The use of mild, low-energy-demanding biocatalytic processes for the transformations of these compounds yields the lowest number of by-products and is the “greenest” alternative. Actually, one of the principles of Green Chemistry is to use catalytic reagents as selective as possible [10].

In this chapter, by combining recent work of our group and literature data, we will review: (1) the applications of lipases and proteases in the synthesis of fatty acid esters of disaccharides and higher oligosaccharides; and (2) the use of transglycosidases for the synthesis of (prebiotic) oligosaccharides from different carbohydrate resources.

17.2 SYNTHESIS OF FATTY ACID SUGAR ESTERS BY LIPASES AND PROTEASES

Sugar esters are nonionic surfactants formed by a carbohydrate moiety as hydrophilic group and one or more fatty acids as lipophilic component. By controlling the esterification degree and the nature of fatty acid and sugar, it is possible to synthesize derivatives with different hydrophile–lipophile balance [11]. Carbohydrate fatty acid esters, synthesized from renewable resources, have a vast number of applications in the food, polymers, cosmetics, oral care, detergent, and pharmaceutical industries [12]. Their properties as antimicrobials for food storage [13], antitumorals [14,15], anticaries [16], and insecticidal [17] indicate their great versatility. Among them, sucrose esters are the most developed carbohydrate esters and are produced at about 4000 MT/y [7].

TABLE 17.1
Carbohydrates Available as Feedstock in Multiton Scale

Polysaccharides	Disaccharides	Monosaccharides	Sugar Derivatives
Starch	Sucrose	Glucose	Sorbitol
Cellulose		Fructose	
Inulin		Xylose	
Xylan			

As sucrose contains eight reactive hydroxyl groups, the degree of substitution and the acylation position on the disaccharide skeleton have a notable effect on its physicochemical properties. Accordingly, monosubstituted sucrose esters are oil-in-water emulsifiers, whereas diesters of long-chain fatty acids and higher esterified derivatives are water-in-oil ones [18]. Regioselective acylation of carbohydrates, i.e., the result of a chemospecificity of one alcohol function among all the others, is an arduous task. Thus, selective chemical acylation requires complex protecting-group methodologies. The regioselectivity can be controlled by structural and electronic factors [19]. It has been demonstrated that the classically accepted view of sucrose nucleophilic reactivity describing the primary 6-OH and 6'-OH as the more reactive positions is not valid anymore, as the reactions can be oriented toward different hydroxyls, depending on the nature of the electrophilic partner and on the reaction conditions and catalysts [19]. Thus, 2-OH selective reactions have been described based on electronic factors [20], primaries 6-OH and 6'-OH have been chemically acylated based on steric hindrance [19], and primary 1'-OH acylation is achieved using proteases as biocatalysts [21].

As stated, sugar esters can be produced using either chemical or biological catalysts. Although they are currently manufactured by base-catalyzed chemical synthesis, the high temperatures required, poor selectivity, and formation of furfurals have focused attention on the more selective enzymatic process [22,23]. Although remarkable work has been done on acylation of monosaccharides as is described elsewhere [24,25], we focus here basically on the acylation of disaccharides and other oligosaccharides, which offer a higher level of structural and functional complexity.

17.2.1 ENZYME REGIOSPECIFICITY

Lipases and carboxylesterases are the “natural” biocatalysts for reactions that involve fatty acids, including sugar ester synthesis. Both types of enzymes belong to the family of carboxylic ester hydrolases (EC 3.1.1), which catalyze the cleavage of ester bonds of lipids and other organic compounds, and are divided into 79 enzymatic families according to the specific bond, moiety, and substrate they hydrolyze [26]. These enzymes are very stable in water and nonpolar organic solvents, and possess regio- and stereospecificity. Unlike carboxylesterases (EC 3.1.1.1), which preferentially hydrolyze water-soluble “simple” esters and triglycerides bearing fatty acids shorter than C₆, lipases (EC 3.1.1.3, also known as triacylglycerol lipases) prefer water-insoluble substrates, typically triglycerides composed of long-chain fatty acids. Apart from lipases and carboxylesterases, serine proteases (EC 3.4.21), specifically those of the subtilisin family, have also been successfully employed in sugar acylation processes [21,27], even with long-chain fatty acids [28].

For the enzyme-catalyzed transesterification of sugars, different regioisomers may be obtained with an appropriate selection of the biocatalyst (Figure 17.1). This is remarkable because, for example, the properties of different sugar monoesters have been reported to vary significantly [29]. While the lipases from *Pseudomonas* sp., *Mucor miehei*, and *Thermomyces lanuginosus* are regiospecific for the hydroxyl 6-OH [30,31], the lipase B from *Candida antarctica* yields a mixture of 6- and 6'-monoesters [13,32]. In contrast, several alkaline proteases catalyze selectively the acylation of the primary 1'-OH at the fructose ring [33–36]. This result is noteworthy because 1'-OH is sterically more hindered than the other primary hydroxyl groups at 6- and 6'-positions. Pedersen's group reported the synthesis of 2-*O*-lauroylsucrose catalyzed by the metalloprotein thermolysin [37]. Recently, several computational studies were performed to explain the differences in regiospecificity [34,38]. One large binding pocket was identified in *C. antarctica* lipase B that accommodates both the sucrose and the acyl moiety of the transition state, whereas in *T. lanuginosus* lipase the binding pocket was found to be smaller, leading to the localization of the two moieties in

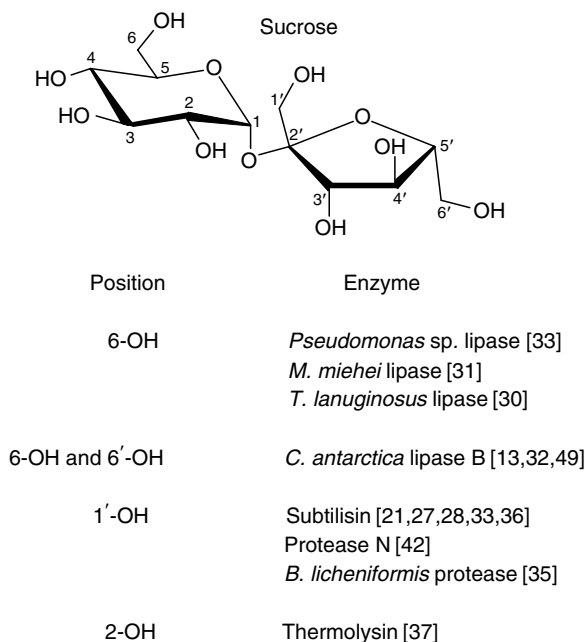


FIGURE 17.1 Regioselectivity displayed by lipases and proteases in the acylation of sucrose.

two distinct pockets (Figure 17.2). This partially explains the broader specificity of *C. antarctica* lipase B [38].

The esterification of other carbohydrates has also been achieved. For example, the 6'-OH group at the nonreducing end of maltose is enzymatically acylated by *T. lanuginosus* lipase and other biocatalysts [13,27,32]. Leucrose, an isomer of sucrose with $\alpha(1\rightarrow5)$ -glucosyl-fructose link, was also tested under similar conditions: the acylation gave rise to three monoesters, although 6-*O*-lauroyl-leucrose represented 92% of the total [39]. Trehalose was acylated at its primary 6-OH by a protease from *Bacillus subtilis* [40]. With the trisaccharide maltotriose, the hydroxyl 6'-OH (at the nonreducing end) was selectively acylated [39]. Subtilisin also esterified the C-1 position of the fructose moiety in the trisaccharide raffinose using different vinyl donors [41].

17.2.2 REACTION MEDIUM

Methodologies for enzymatic carbohydrate acylation (also applicable to other polyhydroxilic compounds) need to find a medium in which a polar reagent (carbohydrate) and a nonpolar acyl donor are soluble and may react in the presence of an appropriate biocatalyst. Polar solvents such as dimethylsulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide, and pyridine were first reported as suitable solvents for sugar solubilization; however, most lipases and carboxylesterases are readily inactivated by these solvents. An alternative for sugar esterification is based on the hydrophobization of the sugar moiety by complexation with phenylboronic acids or by formation of acetals, thus making the substrate more soluble in nonpolar solvents in which most enzymes are active [42].

Some proteases of the subtilisin family are able to catalyze the acylation of sugars in DMF and pyridine [21,28,43]. This fact has been applied to the synthesis of sucrose monomethacrylates, a group of interesting polymerizable compounds [44]. In this context, subtilisins have been engineered by rational and evolution approaches to enhance their activity in polar solvents [45].

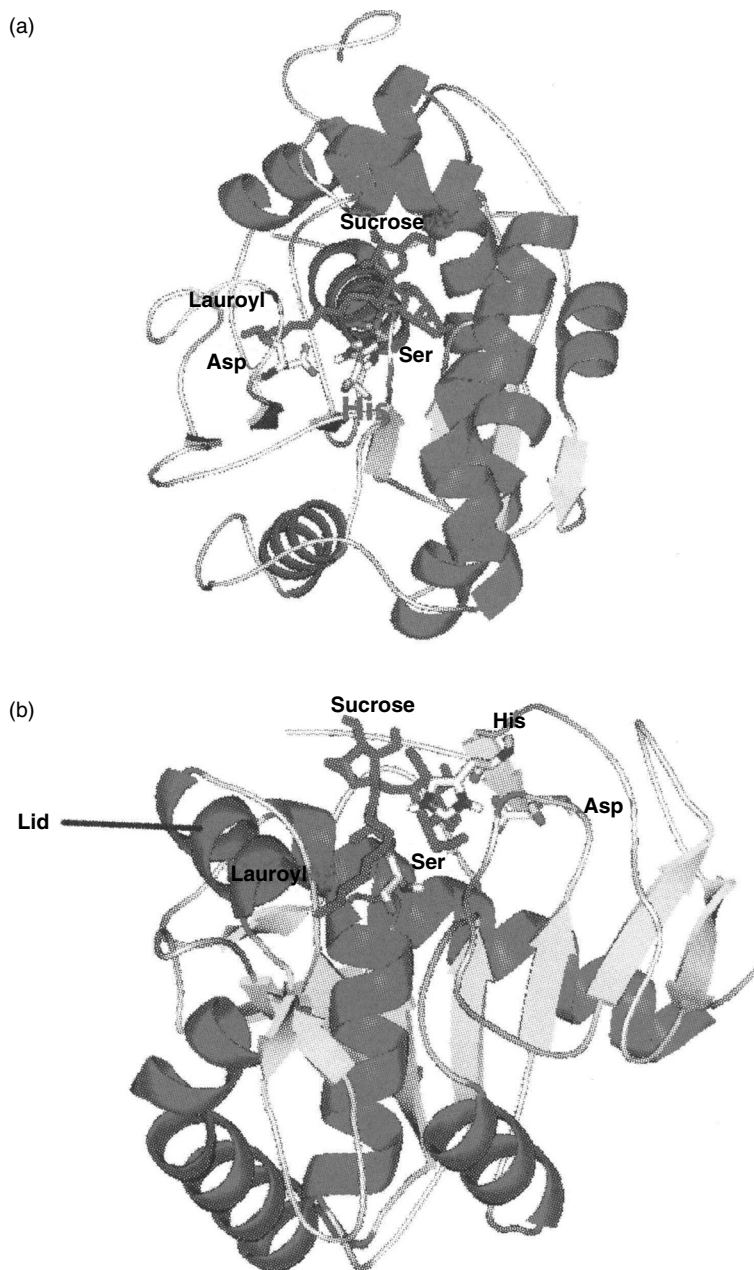


FIGURE 17.2 (See color insert following page 526) Structure of *C. antarctica* (a) and *T. lanuginosus* (b) lipases, docked with lauroylsucrose regioisomers. Both enzymes are characterized by similar overall folds and the same catalytic triad residues and oxyanion hole. (Adapted from Fuentes, G., et al., *Protein Sci.*, 13, 3092, 2004. With permission.)

Another drawback of dipolar aprotic solvents, such as DMSO or DMF, is that some acylating agents, e.g., acid anhydrides and vinyl esters, suffer activation processes that may result in spontaneous nonspecific acylation. Studying the transesterification of sucrose with vinyl laurate in DMSO using immobilized lipases, we unexpectedly found that some carriers

(Celite, Eupergit C) were able to catalyze the process [46]. Furthermore, the simple disodium hydrogen phosphate, used as a buffer during enzyme immobilization, was an efficient catalyst. In the monoester fraction, 2-*O*-acetylsucrose was the major product ($\geq 60\%$), as hydroxyl 2-OH has a special acidity and thus reactivity [19,20].

In an attempt to exploit the potential of lipases for sugar acylation, several processes have been reported using more benign solvents such as ketones or tertiary alcohols that dissolve the carbohydrate only partially. Although this strategy has been fruitful for monosaccharides using *tert*-butyl alcohol [24], 2-methyl-2-butanol [47], or acetone [48], the comparatively lower solubility of oligosaccharides in these solvents resulted in lower conversions [32].

In this context, we developed a simple process for the lipase-catalyzed acylation of sucrose and other carbohydrates [30,39]. The method was based on the presolubilization of sucrose in a polar solvent (DMSO) and its further mixing with a tertiary alcohol [2-methyl-2-butanol – (2M2B)], adjusting the final DMSO content close to 20% (v/v). Our approach of using two miscible solvents as a compromise between sugar solubility and enzyme stability has been successfully followed by other researchers in the acylation of several polyhydroxilic compounds [49–51]. We recently improved the reaction rate by modifying the substrate preparation protocol, namely preincubating sucrose overnight in 2M2B [52]. To emphasize the importance of sugar solubilization, Halling et al. [53,54] observed that in the synthesis of glucose esters, sugar dissolution rate in organic solvents is usually the main limiting parameter for carbohydrate ester production.

Variations in medium polarity also influence the selectivity of enzyme-catalyzed acyl-transfer reactions [55]. To substantiate it, we observed that the percentage of DMSO in the reaction medium changed the ratio of monoester/diester obtained [30,52,56]. In particular, at concentrations $\leq 10\%$ DMSO, the synthesis of diesters was favored, whereas at $\geq 15\%$ DMSO the formation of monoesters was majoritary. This effect occurred with lipase from *T. lanuginosus* and vinyl esters as acylating agents [30], and with methyl palmitate as acyl donor and immobilized lipase B from *C. antarctica* as biocatalyst [52]. In the latter case, Figure 17.3 shows how moving from 0 to 20% DMSO, the molar ratio of monopalmitate/

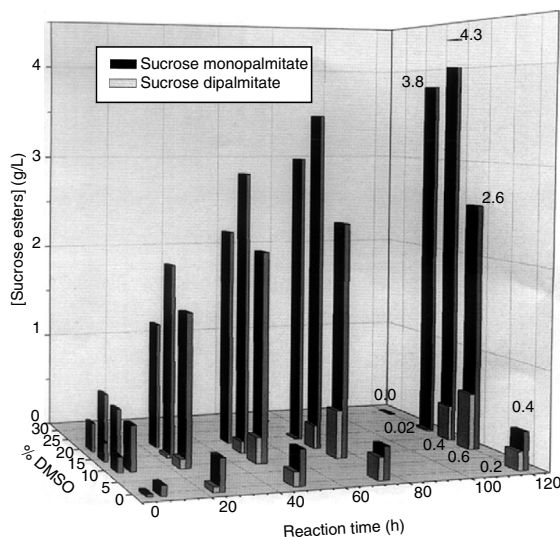


FIGURE 17.3 Effect of DMSO percentage on the ratio of monoester and diester in the transesterification of methyl palmitate with sucrose. Conditions: 0.1 mol/L sucrose, 0.3 mol/L methyl palmitate, 25 g/L lipase B from *C. antarctica* (Novozym 435), 60°C, and 150 rpm. (Adapted from Reyes-Duarte, D., et al., *Biocatal. Biotransform.*, 23, 19, 2005. With permission.)

dipalmitate increased from 65:35 to 99.5:0.5. The effect of solvent polarity on reaction selectivity seems to be related with sucrose solubility [57]: the amount of dissolved sucrose is higher when increasing DMSO content, going from 0.2 g/L in pure 2M2B to 29.5 g/L in 30% DMSO. Consequently, at high DMSO percentage, sucrose competes more efficiently with the monoester formed, which is very soluble in these reaction media for the acyl-enzyme intermediate, resulting in a major presence of monoester. At $\geq 30\%$ DMSO, the reaction rate was negligible, probably due to the inactivation of the lipase by DMSO.

17.2.3 ACYL DONORS

As acylations catalyzed by serine hydrolases take place through the formation of an acyl-enzyme intermediate, the nature of the acyl donor has a notable effect on reactivity [58]. In contrast to hydrolytic reactions, where the nucleophile water is always in great excess, the concentration of nucleophile in acylation processes is always limited; as a result, these reactions are generally reversible in contrast to the irreversible nature of a hydrolytic reaction. The ideal acyl donor should therefore be inexpensive, fast acylating, and completely non-reactive in the absence of the enzyme, but unfortunately such reagents do not exist yet.

For the transformation of inexpensive chemicals, cost is the most important parameter, so simple esters (methyl, ethyl, and glyceryl) are preferred as transesterification reagents. Acylations with these agents are often slow and reversible, and their utility is limited to a narrow range of lipases. This reversibility gives rise to low yields (the thermodynamic equilibrium is commonly not too far from the middle, i.e., $K_{eq} \approx 1$). For this reason, the simple way to increase the rate and yield is to use an excess of acyl donor—but its solubility is limited—or to remove *in situ* the alcohol formed.

A better solution, however, is offered by the use of special acyl donors, which ensure a more or less irreversible reaction. This can be achieved by the introduction of electron-withdrawing substituents (e.g., trihaloethyl esters and oxime esters) [21,59]. Furthermore, if the acyl donor is an enol ester (e.g., vinyl or isopropenyl ester), the reaction yields an enol as the protonated leaving group, which rapidly tautomerizes to acetaldehyde or acetone, respectively (Figure 17.4). These are not nucleophilic, therefore they cannot react with the intermediate acyl-enzyme. In addition, the aldehyde or the ketone formed can be removed by evaporation, thus displacing the equilibrium and making the reaction completely irreversible. Wang et al. [60] demonstrated that the rate of transesterification of hydroxyl-containing compounds with vinyl esters was about 20 to 100 times faster than with other activated esters, and up to 1000 times faster than using methyl or ethyl esters of the same fatty acid.

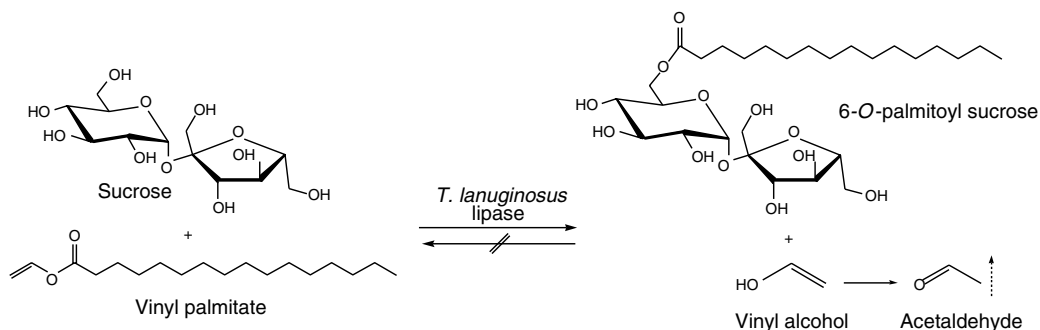


FIGURE 17.4 Transesterification of vinyl palmitate with sucrose catalyzed by *T. lanuginosus* lipase.

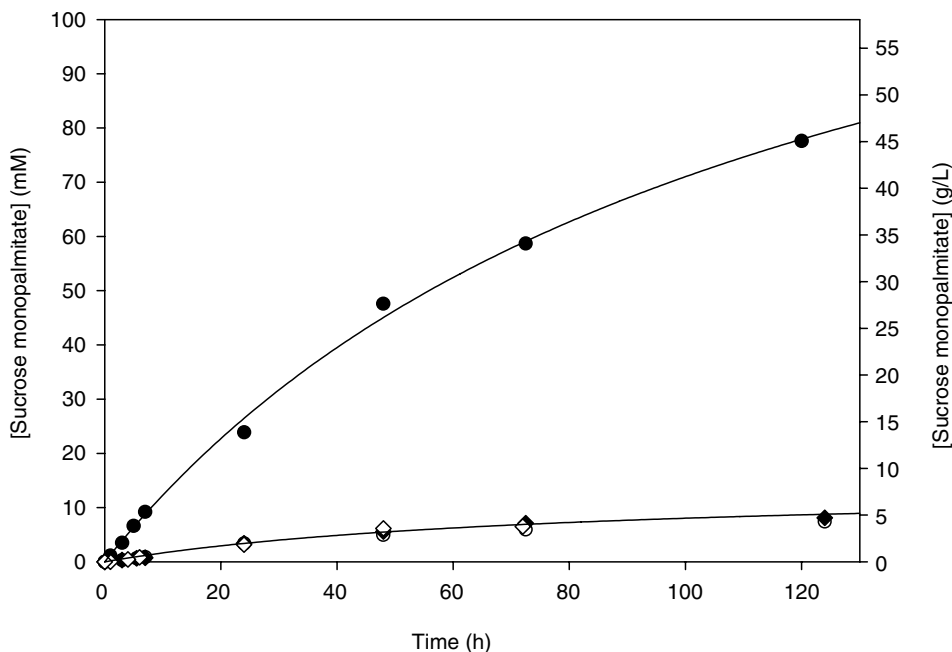


FIGURE 17.5 Effect of the nature of the acyl donor on transesterification. Conditions: 0.1 mol/L sucrose (34 g/L), 0.3 mol/L acyl donor, 2M2B:DMSO 85:15 (v/v), 25 g/L Novozym 435, 60°C, and 150 rpm. Acyl donors: (●) vinyl palmitate; (○) methyl palmitate; (▲) ethyl palmitate; and (◇) palmitic acid. (Adapted from Reyes-Duarte, D., et al., *Biocatal. Biotransform.*, 23, 19, 2005. With permission.)

Due to steric reasons, vinyl esters give higher reaction rates than isopropenyl esters. The major disadvantage of vinyl esters is in the released acetaldehyde. Acetaldehyde may act as an alkylating agent of proteins by forming Schiff's bases in a Maillard-type reaction with amino groups of lysine residues, which can lead to inactivation. In fact, several lipases (e.g., *C. rugosa* or *Geotrichum candidum*) lose most of their activity when exposed to acetaldehyde [61]. The addition of molecular sieves (4 Å) to the medium to trap acetaldehyde seems to protect against inactivation [62]. However, considering the low boiling point of acetaldehyde (21°C), it can be removed by simply performing the reaction in an open vessel. Vinyl acetate is routinely employed in different types of transesterifications and, for the past few years, vinyl esters of longer saturated fatty acids (C₈–C₁₈) are also commercially available at a reasonable price. Figure 17.5 shows that the use of vinyl palmitate gives rise to a productivity one order of magnitude higher than that obtained with other activated (methyl palmitate or ethyl palmitate) or nonactivated (palmitic acid) acylating agents [52].

17.3 SYNTHESIS OF OLIGOSACCHARIDES BY TRANSGLYCOSIDASES

It is generally accepted that an oligosaccharide is a carbohydrate consisting of 2 to 10 monosaccharide residues linked by *O*-glycosidic bonds [63,64]. The development of efficient and scalable synthetic processes for addressing oligosaccharides in the food (e.g., prebiotics, sweeteners, stabilizers, bulking agents) and pharmaceutical industries (as therapeutics in prevention of infection, neutralization of toxins, and immunotherapies) is of great interest [65,66].

Considering the structural diversity of oligosaccharides (3 different amino acids would allow the synthesis of only 6 different peptides, while 3 different hexopyranose moieties would yield up to 720 trisaccharides), the stereo- and regioselectivity of enzymes are considered a valuable alternative to chemical synthesis, which needs complex protection and deprotection steps for the preparation of structurally well-defined oligosaccharides. Actually, enzymatic processes are preferred in the food industry for the production of most important oligosaccharides.

In vivo synthesis of glycosidic bonds is performed by glycosyltransferases (EC 2.4.) [67]. These enzymes catalyze the coupling (by a transfer reaction) of a glycosyl donor to an acceptor molecule forming a new glycosidic bond with regio- and stereoselectivity. According to the nature of the sugar residue transferred, glycosyltransferases are divided into hexosyltransferases (EC 2.4.1.), pentosyltransferases (EC 2.4.2.), and those transferring other glycosyl groups (EC 2.4.99.).

Depending on the nature of the donor molecule, glycosyltransferases are classified into three main mechanistic groups: (1) Leloir-type glycosyltransferases, which require sugar nucleotides (e.g., UDP-glycosyltransferase); (2) non-Leloir glycosyltransferases, which use sugar-1-phosphates (e.g., phosphorylases); and (3) transglycosidases, which employ nonactivated oligosaccharides (e.g., sucrose, starch) as glycosyl donors. A distinctive feature of transglycosidases, compared with Leloir and non-Leloir glycosyltransferases, is that they also display some hydrolytic activity, which can be regarded as a transfer of a glycosyl group from the donor to water. It is noteworthy that, in terms of reaction mechanism, transglycosidases belong to the same group as that of glycosidases (3.2.), a group of hydrolases that catalyze with exquisite stereoselectivity the hydrolysis of glycosidic bonds in oligo- and polysaccharides. According to the Henrissat classification, which is based on amino acid sequence comparisons, transglycosidases and glycosidases constitute the “glycoside hydrolase family (GH family)”, with more than 2500 enzymes [68].

In vitro oligosaccharide synthesis can be performed with glycosyltransferases and glycosidases. There are several problems associated with the use of glycosyltransferases of Leloir and non-Leloir type: (1) the requirement of sugar nucleotides or sugar phosphates as substrates, whose synthesis is rather difficult and expensive; (2) the inhibitory effect of the nucleotide phosphate released; and (3) the limited availability of these enzymes [69]. Glycosidases are widely employed for oligosaccharide synthesis, as under appropriate conditions the normal hydrolytic reaction can be reversed toward glycosidic bond synthesis [70]. This can be achieved by thermodynamic control (using low-water concentrations) or by kinetic control (using activated glycosyl donors at high concentrations). Despite the broad specificity of glycosidases and their availability, the application of these catalysts is often limited by low yields and poor regioselectivity [4].

In this context, transglycosidases constitute the ideal biocatalysts for oligosaccharide synthesis *in vitro*, because they do not require special activated substrates, as they directly employ the free energy of cleavage of disaccharides (e.g., sucrose) or polysaccharides (e.g., starch) [71]. Transglycosidases present the same mechanism as retaining glycosidases, resulting in net retention of anomeric configuration. The active site contains two carboxylic acid residues, located at ~5.5 Å apart: one acting as a nucleophile and the other as an acid and base catalyst (Figure 17.6). The reaction proceeds by a double-displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed by the attack of the deprotonated carboxylate to the anomeric center of the carbohydrate with concomitant C–O breaking of the scissile glycosidic bond [72]. The first step is assisted by the carboxylic residue acting as general acid. The second step is the attack of a nucleophile to the glycosyl-enzyme intermediate, which is assisted by the conjugate base of the second carboxyl residue.

The nucleophiles (H₂O) and the acceptor (carbohydrate) compete for the glycosyl-enzyme intermediate (Figure 17.6). When the nucleophile is H₂O, the enzyme acts as a hydrolase; when the sugar is the nucleophile, the enzyme acts as a transferase. The transferase to

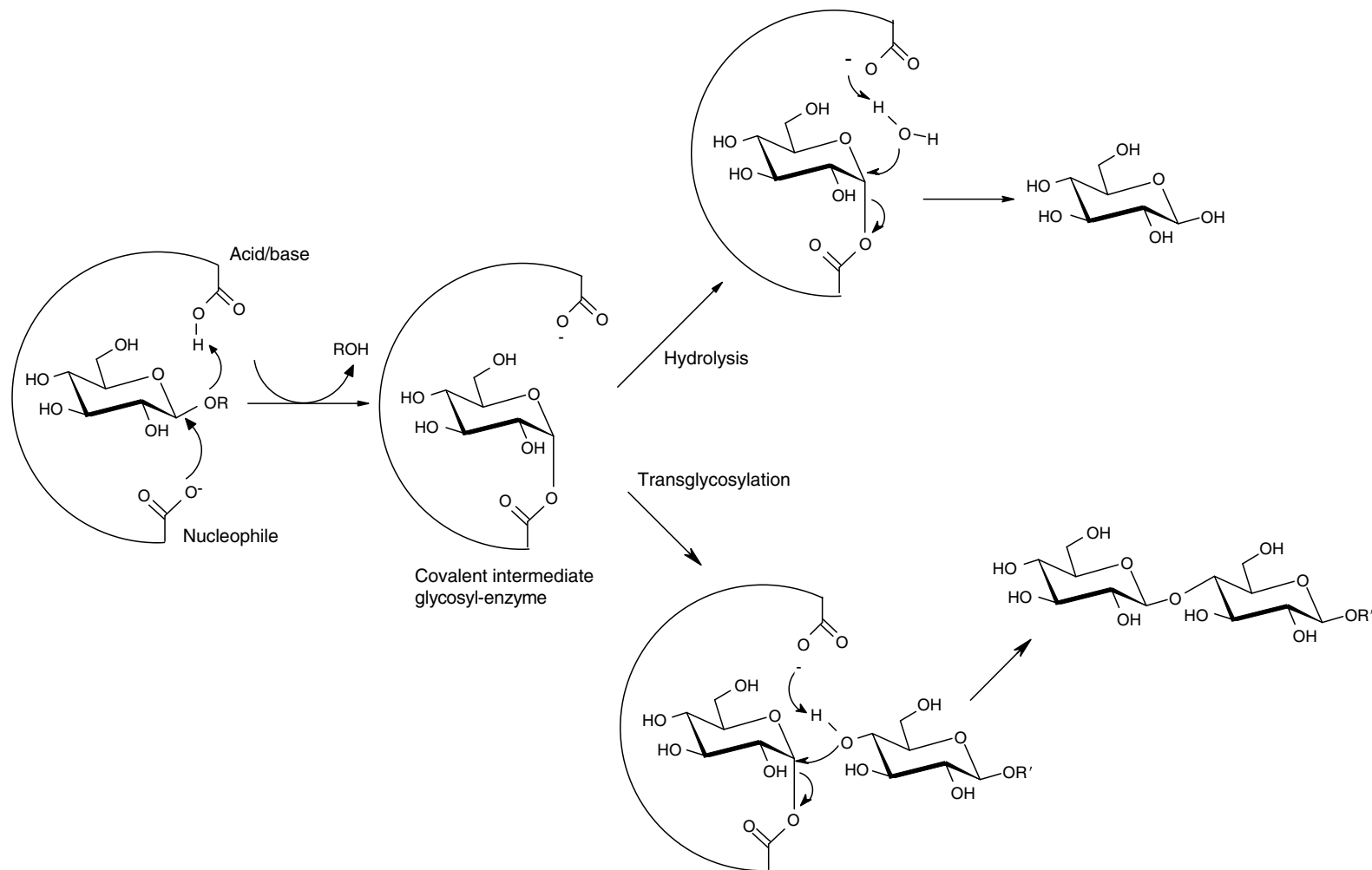


FIGURE 17.6 Mechanism of the action of retaining glycosidases and transglycosidases.

hydrolase ratio depends on two main factors: (1) the concentration of acceptor (high concentrations must be used to enhance glycosyl transfer); and (2) the intrinsic enzyme properties. A transglycosidase will be considered efficient if it possesses significant ability to bind the acceptor and to exclude H₂O.

As a consequence of the progress in the understanding of the structures and catalytic mechanisms involved in the enzymatic synthesis of glycosidic bonds, a group of novel mutants—called glycosynthases—were developed by site-directed mutagenesis of glycosidases [73]. The glycosynthase concept was introduced in 1998 by Whitters' group on an exoglucosidase [74], and extended to endo-glycosidases by Planas' group [75]. A glycosynthase is a specifically mutated retaining glycosidase in which substitution of the catalytic carboxyl nucleophile by a noncatalytic residue (Ala, Gly, or Ser) renders a hydrolytically inactive enzyme, but is yet able to catalyze the transglycosylation of activated glycosyl fluoride donors (having the opposite anomeric configuration of that of normal substrates of the parental wild-type enzyme). The yield obtained with glycosynthases reaches 95 to 98% in some cases [69]. The impressive amount of glycosidases available clearly indicates that the potential biodiversity of glycosynthases is still largely unexplored, and new applications of these enzymes will emerge in the near future [76].

17.3.1 TRANSFRUCTOSIDASES

Many microbial organisms and about 12% (i.e., ~40,000 species) of the higher plants build carbohydrate storage based on fructans, polymers formed by β-D-fructofuranose units with a terminal D-glucose. The fructosyl moieties are β(2→6)-linked in the case of levan or β(2→1)-linked in the case of inulin, and the enzymes responsible for fructan synthesis are referred to as levansucrases and inulosucrases, respectively [77]. They belong to the glycansucrases family, a group of transglycosidases acting on sucrose and utilizing it as the sole energy source for oligo- and polysaccharide synthesis (Table 17.2). Glycansucrases are subdivided into glucansucrases and fructansucrases depending on the transferred monosaccharide.

An interesting feature of several glycansucrases is their ability to catalyze the synthesis of low-molecular-weight (LMW) oligosaccharides from sucrose when efficient acceptors are

TABLE 17.2
Polysaccharides Synthesized by Glycansucrases

Polysaccharide	Bond Formed	Branching	Enzyme	EC Number	Source Microorganism	Group Transferred
Dextran	α-(1→6)	α-(1→2) α-(1→3)	Dextransucrase (Glucansucrase)	2.4.1.5	<i>Leuconostoc mesenteroides</i> <i>Streptococcus strains</i>	Glucosyl
Mutan	α-(1→3)	α-(1→6)	Mutansucrase (Glucansucrase)	2.4.1.5	<i>Streptococcus strains</i>	Glucosyl
Amylose	α-(1→4)	—	Amylosucrase	2.4.1.4	<i>Neisseria polysaccharea</i>	Glucosyl
Alternan	α-(1→3) alternating with α-(1→6)	α-(1→3)	Alternansucrase	2.4.1.140	<i>Leuconostoc mesenteroides</i>	Glucosyl
Inulin	β-(2→1)	β-(2→6)	Inulosucrase	2.4.1.9	<i>Leuconostoc citreum</i> <i>Lactobacillus reuteri</i>	Fructosyl
Levan	β-(2→6)	β-(2→1)	Levansucrase	2.4.1.10	<i>Bacillus subtilis</i> <i>Rahnella aquatilis</i>	Fructosyl

added to the reaction medium. The addition of acceptor causes a decrease in the amount of polymer formed, as both reactions are competitive [78].

17.3.1.1 Synthesis of Inulin-Type Fructooligosaccharides

Short-chain fructooligosaccharides (FOS) of the inulin type constitute one of the groups of prebiotic oligosaccharides most established in the world [79]. Prebiotic agents are food ingredients that are potentially beneficial to the health of the consumers. Prebiotics escape enzymatic digestion in the upper gastrointestinal tract and enter the colon without any change in their structure. None are excreted in the stools, indicating that they are fermented by colonic flora so as to give a mixture of short-chain fatty acids (acetate, propionate, and butyrate), L-lactate, carbon dioxide, and hydrogen [80]. By selectively stimulating beneficial intestinal bacterial genera such as *Bifidobacterium* and *Lactobacillus*, they may have the following implications for health: (1) potential protective effects against colorectal cancer and infectious bowel diseases by inhibiting putrefactive (*Clostridium perfringens*) and pathogen (*Escherichia coli*, *Salmonella*, *Listeria*, and *Shigella*) bacteria, respectively; (2) improvement of glucid and lipid metabolisms; (3) fiber-like properties by decreasing the renal nitrogen excretion; and (4) improvement in the bioavailability of essential minerals [81–85]. FOS are also noncariogenic and have a sweet taste of about 40 to 60% that of sucrose.

FOS of the inulin type are fructose oligomers with a terminal glucose group, in which 2–4 fructosyl moieties are linked through $\beta(1\rightarrow2)$ -glycosidic bonds [86]. Their structural formula is α -D-glucopyranosyl-(1 \rightarrow 2)-[β -D-fructofuranosyl-(1 \rightarrow 2)-] $_n$ (GF $_n$). Commercial FOS are mainly composed of 1-kestose (GF $_2$), nystose (GF $_3$), and 1^F-fructofuranosyl-nystose (GF $_4$).

FOS are industrially produced through fructosyl transfer from pure sucrose using a fungal enzyme (Figure 17.7) [87]. FOS-synthesizing enzymes are produced by many higher plants (e.g., asparagus, chicory, onion, Jerusalem artichoke) and microorganisms, especially fungi (e.g., *Aureobasidium pullulans*, *Aspergillus niger*, *A. oryzae*) [86,88–90]. Inulosucrases that do not produce FOS but inulin polymers have also been isolated from plants and fungi [91].

FOS-producing enzymes belong to families 32 and 68 in the Henrissat classification [68]. The inclusion of FOS-producing enzymes in the group of glycosidases (β -fructofuranosidases, EC 3.2.1.26) or fructosyltransferases (transfructosidases, EC 2.4.1.9) still remains controversial. However, the mechanism displayed by both groups of enzymes is essentially the same (Figure 17.6), and the assignment of a particular enzyme as β -fructofuranosidase or transfructosidase should be based on the transferase to hydrolysis ratio. Only a few of these enzymes have a significant level of transfructosylating activity to make them useful for industrial applications. Recently, several FOS-synthesizing enzymes from *Aspergillus* spp. have been purified and characterized [92], and the first three-dimensional structure of a β -fructofuranosidase, that of *Thermotoga maritima*, has been resolved [93].

The maximal FOS production for a particular enzyme depends on the relative rates of transfructosylation and hydrolysis [94], which means that the synthesis of oligosaccharides by transglycosidases is kinetically controlled. Consequently, the FOS concentration reaches a maximum (Figure 17.8) that may be substantially higher than the equilibrium concentration [95]. The time required to get the maximum FOS production depends inversely on the amount of the enzyme; however, the FOS concentration at this maximum is not affected by the amount of biocatalyst.

Ghazi et al. [95] obtained a maximum FOS production of 61.5% (w/w) in 24 h, referred to total carbohydrates in the mixture, using an immobilized transfructosidase (Figure 17.8). The weight ratio of 1-kestose/nystose/1^F-fructofuranosylnystose was 6.2/3.7/0.1. At equilibrium (150 h), the FOS production was slightly lower (57 to 58%), although the product distribution changed notably. At this stage the rest of the carbohydrates were glucose (29 to 31%), sucrose

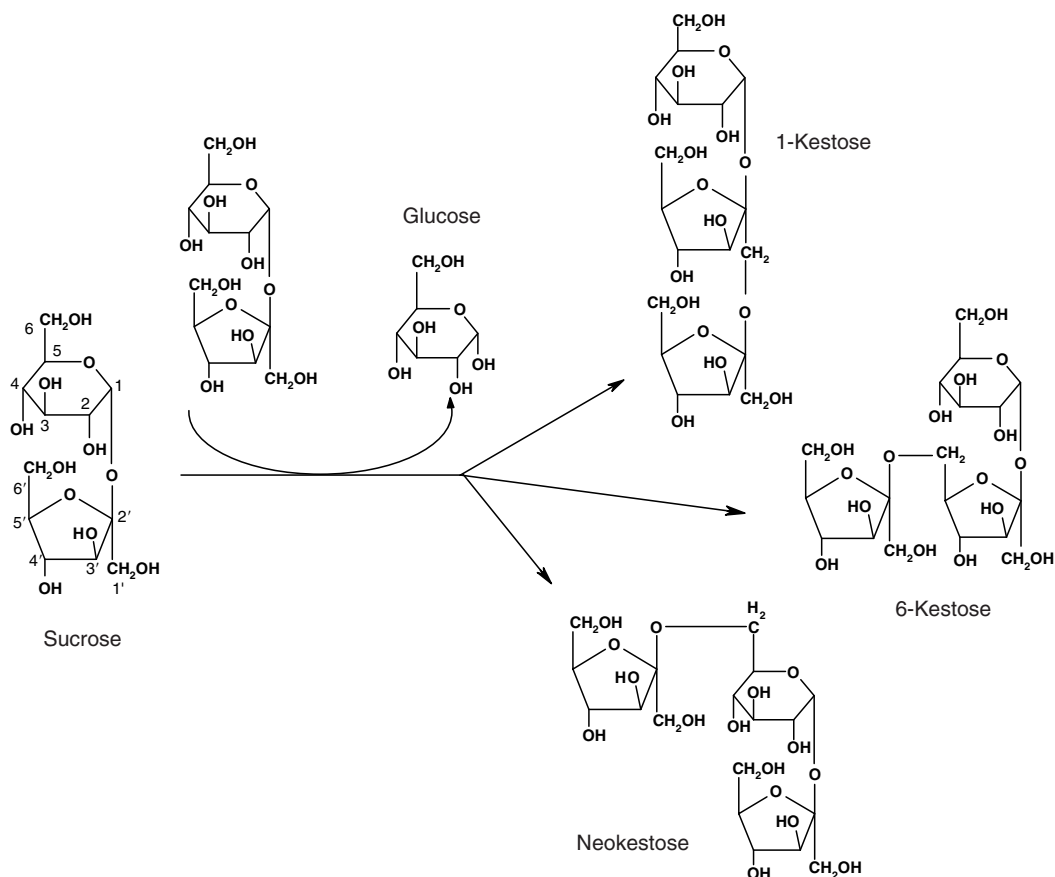


FIGURE 17.7 Reaction scheme for the synthesis of inulin-type fructooligosaccharides, neo-FOS, and ⁶F-type FOS catalyzed by transfructosidases.

(9.5 to 10.5%) ⁶F-type and fructose (2 to 3%). Similarly, yields of FOS have been reported with other immobilized transfructosidases [97,98].

Levansucrases catalyze the synthesis from sucrose of levan, a polymer with applications in food, cosmetic, and pharmaceutical industries. In addition to levan formation, levansucrase concomitantly produces FOS of the inulin type [99–101]. Levansucrases also catalyze other transfructosylation reactions in the presence of acceptors such as methanol [102], glycerol [103], and disaccharides [104]. Levansucrases fall into glycoside hydrolase (GH) family 68 in the “carbohydrate-active enzymes” database [68]. Several studies have focused on elucidating the structure and the function of these enzymes. The crystal structure of *B. subtilis* levansucrases was recently solved by Meng and Fütterer [105] at 1.5 Å resolution, and shows a rare fivefold propeller. Site-directed mutants of the three putative catalytic residues of the *Lactobacillus reuteri* 121 levansucrase and inulosucrase (the catalytic nucleophile, the general acid–base catalyst, and the transition state stabilizer) have been recently obtained [106].

17.3.1.2 Synthesis of ⁶F-Type and Neo-Fructooligosaccharides

Neo-fructooligosaccharides (neo-FOS) consist mainly of neokestose (neo-GF2) and neonytose (neo-GF3), in which a fructosyl unit is $\beta(2\rightarrow6)$ bound to the glucose moiety of sucrose and 1-kestose, respectively (Figure 17.7).

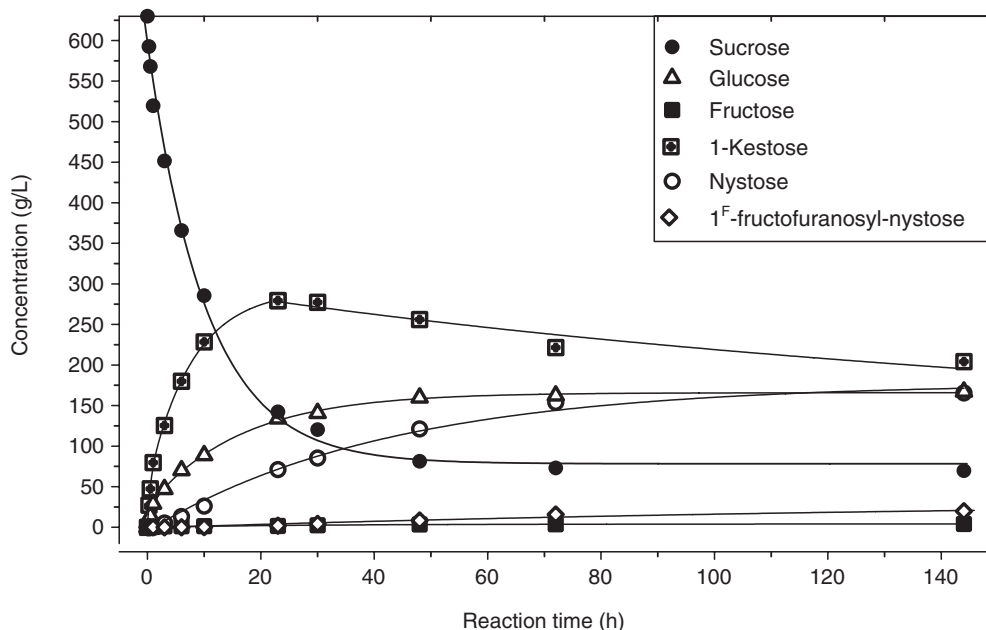


FIGURE 17.8 Time course of the fructooligosaccharides production catalyzed by a transfructosidase from *Aspergillus aculeatus* (Pectinex Ultra SP-L). Experimental conditions: 630 g/L sucrose, 0.3 U/ml, 50 mM sodium acetate buffer (pH 5.4), and 60°C. (Adapted from Ghazi, I., et al., *J. Mol. Catal., B Enzym.*, 35, 19, 2005. With permission.)

Grizard and Barthomeuf [107] were the first in reporting the enzymatic synthesis of neo-FOS using a transfructosylating activity present in a commercial enzyme from *A. awamori*. The neo-FOS yield reached a maximum of 50% (w/w) based on the total weight of carbohydrates in the reaction mixture. Cultures of the astaxanthin-producing yeast *Xanthophylomyces dendrorhous* accumulated neokestose as a major transfructosylation product when growing on sucrose [108,109]. In addition, neokestose occurs only as a minor transfructosylating product of whole-cells or enzymes from various plants, yeasts (e.g., *Saccharomyces cerevisiae*), and some filamentous fungi [110]. Investigations using human feces as inoculum *in vitro* have demonstrated that neokestose has prebiotic effects that surpass those of commercial FOS [111].

Short-chain ⁶F-type FOS have also received some attention (Figure 17.7). In fact, linear and branched β -(2,6)-linked FOS (the first is 6-kestose) occur naturally in various food products [112]. However, the enzymatic synthesis of ⁶F-type FOS has been scarcely reported. Bekers et al. [113] determined the presence of the trisaccharides 1-kestose, neokestose, and 6-kestose in the fructans syrup obtained with a levansucrase from the ethanol-producing bacteria *Zymomonas mobilis*.

17.3.2 TRANSGLUCOSIDASES

17.3.2.1 Glucansucrases

Several bacteria excrete a range of transglucosidases called glucansucrases that utilize sucrose as the sole energy source to polymerize its glucosyl moiety (Table 17.2). Glucansucrases belong to family 70 of the GH family in the Henrissat classification [68].

Dextranases and glucanases from the mutans streptococci are the most important members of this group. Glucanases from mutans streptococci (EC 2.4.1.5) are able to synthesize extracellular polysaccharides (glucans) from dietary sucrose *in situ*, which serve as adherence sites for streptococci [16,114]. In the protected environment conferred by the glucans, the mutans streptococci and other microorganisms form a stable and protected community (dental plaque) and release sufficient quantities of metabolic acids to demineralize tooth enamel and initiate dental caries. Mutans streptococci secrete at least three glucosyltransferases: two synthesize α -1,6-linked water-soluble glucans (dextrans), which differ in glucan affinity and degree of α -1,3 branching, while the third synthesizes an α -1,3-linked water-insoluble glucan (mutan) [115].

Dextranases (sucrose: 1,6- α -D-glucan 6- α -D-glucosyltransferase, EC 2.4.1.5) are closely related to glucanases from mutans streptococci. Dextranases are produced by different *Leuconostoc mesenteroides* strains and catalyze the synthesis, from sucrose, of α (1 \rightarrow 6)-linked glucose polymers called dextrans, releasing fructose [116]. In the presence of other compounds, generally mono-, di-, and short oligosaccharides, the synthesis of acceptor products may occur [117]. The three reactions catalyzed by dextranase are: (1) polymerization of the glucose moiety of sucrose; (2) glucose transfer to acceptors; and (3) sucrose hydrolysis, which are competitive, indicating that they take place at the same active site [118].

The regioselectivity displayed by dextranases is highly strain-dependent [119]. With dextranase from *L. mesenteroides* NRRL B-512F, the synthesis of oligosaccharides with α (1 \rightarrow 6)-linked glucose moieties is observed [120]; however, dextranase from the strain B-1299 is also able to form α (1 \rightarrow 2) linkages [121,122]. Due to the nature of the linkages formed between the glucose moieties, most of the products synthesized by dextranase display prebiotic properties.

Numerous sugars can act as acceptors of dextranase, which can be classified as (1) strong acceptors, e.g., maltose, which enhance the reaction rate (measured as fructose released) and strongly inhibit the synthesis of dextran; and (2) weak acceptors, e.g., fructose, which have an inhibitory effect on glucan formation, but yield a low amount of acceptor products [116]. Figure 17.9 shows how increasing concentrations of a strong acceptor (i.e., α -D-methyl glucoside) correlate with higher inhibition of dextran formation [123].

The acceptors can also be classified according to the acceptor products obtained: (1) those that give a homologous series of oligosaccharides, each differing one from the other by one glucose residue (e.g., isomaltose); and (2) those that only form a single acceptor product containing one glucose residue more than the acceptor [118]. The latter is the case of fructose, which is a major product in all dextranase-catalyzed reactions. Fructose yields leucrose (5-*O*- α -D-glucopyranosyl-D-fructopyranose) and a minor product, isomaltulose (4-*O*- α -D-glucopyranosyl-D-fructofuranose), in which fructose is in the furanose form. The leucrose synthesis process becomes particularly important at the final stages of the glucan synthesis reaction as the fructose concentration is high [124].

With several acceptors such as glucose, methyl 1-*O*- α -D-glucopyranoside, maltose or isomaltose, the glucose unit from sucrose is transferred to the C-6 hydroxyl of the monosaccharide or to the 6-OH of the nonreducing end glucose of the disaccharide. This yields a series of isomaltodextrins with degree of polymerization from 2 to 7 attached to the acceptor. Isomaltooligosaccharides constitute an important group of prebiotics that are also useful as immunostimulants and anticaries agents [125,126].

Using dextranase from *L. mesenteroides* B-1299, glucooligosaccharides containing α (1 \rightarrow 2) are synthesized, which exhibit particular prebiotic properties [127,128]. Very recently, Boucher et al. [129] demonstrated that a diet supplemented with glucooligosaccharides containing α (1 \rightarrow 2) linkages may regulate the carbohydrate metabolism, and it may be useful for patients exhibiting loss of insulin sensitivity. In addition, they are capable of promoting

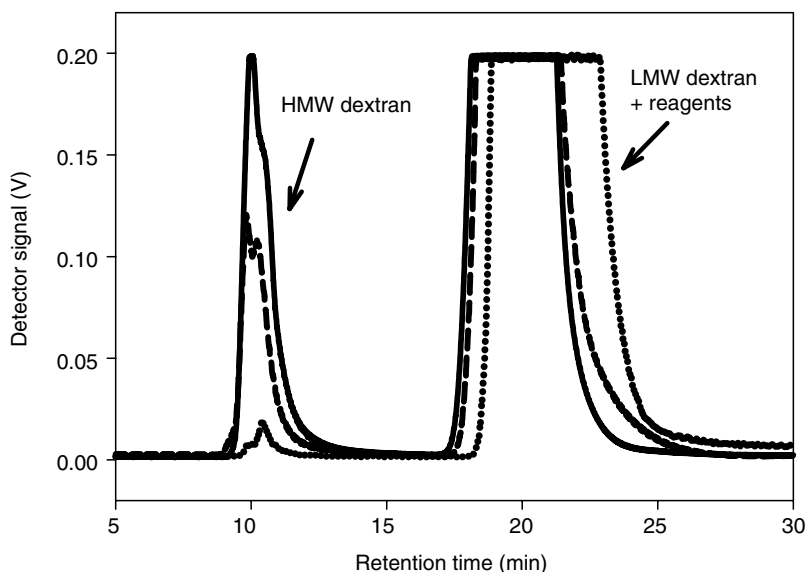


FIGURE 17.9 Size exclusion chromatography analysis of high-molecular-weight (HMW) dextrans and low-molecular-weight (LMW) oligosaccharides synthesized by dextransucrase B-512F, at different mass ratios sucrose:methyl α -D-glucopyranoside, 100:100 (g/L) (—), 100:400 (g/L) (---), and 100 g/L sucrose in absence of acceptor (....). Reaction time: total consumption of sucrose (24 to 50 h). Experimental conditions: 100 g sucrose/L, 0.3 U/ml, 30°C, and pH 5.4. (Adapted from Gómez de Segura, A., et al., *Food Technol. Biotechnol.*, 42, 337, 2004. With permission.)

the development of the beneficial cutaneous flora in detriment of the undesirable microorganisms, either pathogenic or those associated with infections. Based on the acceptor reaction with maltose, dextransucrase from *L. mesenteroides* B-1299 is being employed for producing 50 MT/y of nondigestible glucooligosaccharides containing $\alpha(1\rightarrow2)$ bonds for the dermocosmetic industry [130].

Cellobiose gives an unusual series in which the first product is 2- α -D-glucopyranosyl cellobiose with glucose attached to the C-2 of the reducing-end moiety. With lactose, a cellobiose analog, only the first acceptor product is formed, but with similar regioselectivity [131]. Nonconventional acceptors such as D-sorbitol, α -D-glucosyl-1,6-D-sorbitol, α -D-glucosyl-1,6-D-arabonic acid, and D-glucal can also be glucosylated with dextransucrase [132].

17.3.2.2 Cyclodextrin Glucosyltransferases

Cyclodextrin glucanotransferases (CGTases, EC 2.4.1.19) are a group of transglucosidases that belong to the family 13 of GH [68], called α -amylase family. CGTases catalyze the formation of cyclodextrins (CDs) from starch by way of an intramolecular transglucosylation reaction (cyclization), in which part of the $\alpha(1\rightarrow4)$ -amylose chain is cyclized by formation of an additional $\alpha(1\rightarrow4)$ -glucosidic bond (Figure 17.10). CDs possess a hydrophilic exterior and a hydrophobic cavity, and have numerous applications in food, pharmaceutical, chemical, and cosmetic industries [133]. CGTases usually produce a mixture of α , β , and γ -CDs (containing six, seven, and eight α -D-glucose units, respectively), and are accordingly classified as α , β , and γ -CGTases [134]. For example, the commercially available CGTase from *Thermoanaerobacter* sp., whose optimum temperature is 85°C at pH 5.5 [135], is able to convert about 30% of a 25% (w/v) starch-slurry into a mixture of CDs, with a ratio α : β : γ of 3:5:2 [136].

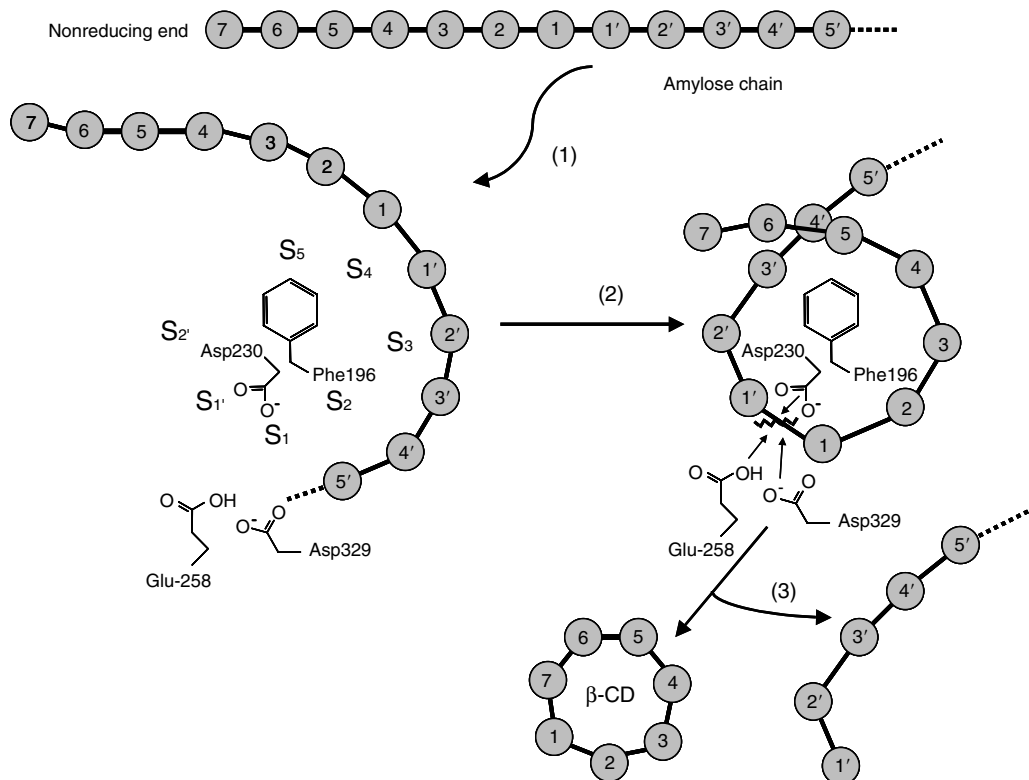


FIGURE 17.10 Cyclodextrin synthesis by CGTases. (1) The amylose chain is attached to the CGTase active site with the help of the cyclization axis (generally an aromatic residue, either Phe or Tyr). (2) The 3-carboxylic acid residues exert a combined attack between subsites S_1 and S_1' . (3) The reducing end of amylose is released from active site. Afterward, the amylose nonreducing end acts as an acceptor (placing at subsites S_1' and S_2') forming the new glycosidic bond and releasing the cyclodextrin.

Besides the cyclization process, CGTases catalyze intermolecular transglucosylation using CDs or oligosaccharides as glucosyl donors; these reactions are referred to as coupling and disproportionation, respectively. In addition, CGTases catalyze the hydrolysis of starch and maltooligosaccharides [137]. The three-dimensional structure elucidation and the biochemical characterization of site-directed mutants have yielded a detailed insight into the mechanism of the reactions catalyzed by CGTases [138]. CGTase can be changed into a starch hydrolase with high exospecificity by hampering substrate binding at the remote donor substrate binding subsites [139]. In addition, CGTase was transformed into a starch hydrolase by directed evolution [140]. Chemical modification of certain residues of CGTase has also allowed to increase transglycosylation [141] or hydrolysis [142] activities. When these acceptors are present in the reaction mixture, they inhibit the formation of cyclodextrins because the glucosyl moiety of the donor (starch) is deviated toward the acceptor reaction (Figure 17.11).

The acceptor specificity of CGTase is rather broad. It is able to use various carbohydrates and related compounds as acceptors by way of intermolecular transglucosylation reactions, coupling or disproportionation. The transglucosylation capability of CGTase seems to be very dependent on the enzyme source [143].

To act as a CGTase acceptor, a carbohydrate must have a D-glucopyranose structure (chair form) with equatorial hydroxyl groups at C-2, C-3, and C-4 [134]. Among monosaccharides, D-glucose, D-xylose, and L-sorbose are the most effective acceptors. CGTase

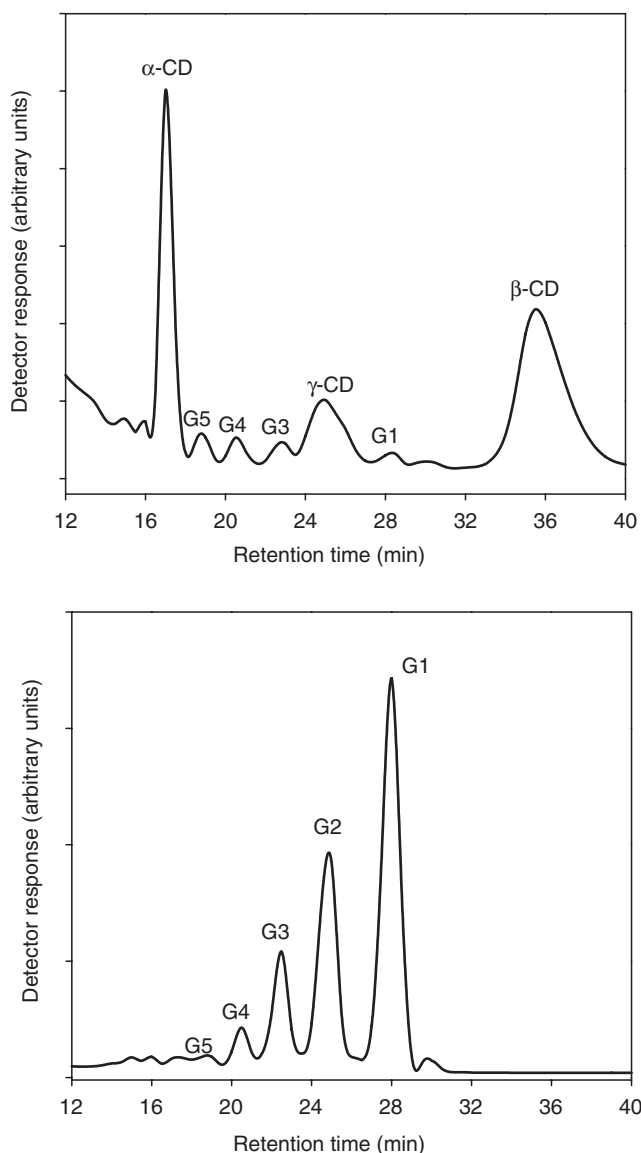


FIGURE 17.11 HPLC chromatograms of products synthesized by CGTase in the absence (top) and in the presence of D-glucose (bottom). Conditions: 0.7 $\mu\text{g/ml}$ *Thermoanaerobacter* CGTase, 10% (w/v) soluble starch, 20% (w/v) D-glucose, 60°C, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM CaCl_2 , and incubation for 48 h. (Adapted from Martin, et al., *Biocatal. Biotransform.*, 19, 21, 2001. With permission.)

transfers glucosyl residue to the hydroxyl group at the C-4 of D-glucose and D-xylose, but to the 3-OH of L-sorbose. On the contrary, D-galactose, D-ribose, D-mannose, D-arabinose, and D-fructose—which do not present the mentioned configuration of 2-OH, 3-OH, and 4-OH—give rise to very low or even negligible yields of acceptor products.

The transglycosylation to disaccharides containing a nonreducing end glucose residue occurs mainly or exclusively at the C-4 hydroxyl group of this glucose moiety. Nakamura

TABLE 17.3
Examples of Acceptors That Work with Transfructosidases and Transglucosidases

Type of compound	Acceptor	Enzyme	References
Carbohydrates	Monosaccharides, Disaccharides	CGTase, dextranucrase, transfructosidases	104,145,151–153
Sugar alcohols	Myo-inositol, Sorbitol, Lactitol, Xylitol, Maltitol	CGTase	147,154
Functionalized sugars	Alditols, Aldosuloses, Sugar acids, Glycals, Alkyl saccharides, Fructose dianhydride	Dextranucrase	155
Glycosides	Rutin, Salicin	CGTase	143,156
Vitamins	Ascorbic acid	CGTase	146,157
Simple alcohols	Methanol	Levansucrase	102
Polyols	Trimethylolpropane, Pentaerythritol	CGTase	158
	Glycerol	Levansucrase	103
Flavonoids	Hesperidin, Naringin, Catechin	CGTase	150,159,160

et al. [144] demonstrated that maltose and cellobiose were better acceptors than glucose for the transglycosylation reaction of CGTase. With maltose or glucose as acceptors and starch as donor, linear malto-oligosaccharides (MOS) composed of α -D-glucose residues linked by $\alpha(1\rightarrow4)$ -glycosidic bonds are produced [145]. A homologous series of MOS is obtained, as shown in Figure 17.7. The degree of polymerization of the oligosaccharides formed can be modulated varying the starch to acceptor ratio. The higher the acceptor concentration, the higher will be the selectivity to LMW oligosaccharides.

It is well documented that CGTase has a higher affinity to disaccharides compared with monosaccharides [143]. This suggests that the acceptor-binding site can recognize at least two glucopyranose moieties. Disaccharides such as isomaltose, gentiobiose, turanose, maltulose, isomaltulose, cellobiose, and sucrose act as good CGTase acceptors, yielding the corresponding oligosaccharides. A steric factor possibly plays a major role in diminishing the acceptor capacity of maltooligosaccharides larger than maltose (e.g., maltotriose).

Other hydroxyl-compounds, such as glycosides, sugar alcohols, vitamins, flavonoids, etc., have been also reported to act as acceptors, in many cases with high efficiency [146–148]. Glucosylation often results in new stability and solubility properties [149,150]. Table 17.3 summarizes the wide nature of acceptors that can be “recognized” by transglycosidases.

ACKNOWLEDGMENTS

This work was supported by the European Union (Project MERG-CT-2004-505242) and the Spanish CICYT (Projects BIO2003-02473 and BIO2004-03773-C04-01). M.F. thanks the Spanish Ministerio de Ciencia y Tecnología for its support.

REFERENCES

1. Lichtenthaler, F.W. and Peters, S., Carbohydrates as green raw materials for the chemical industry, *C. R. Chim.*, 7, 65, 2004.
2. Jenck, J.F., Agterberg, F., and Droescher, M.J., Products and processes for a sustainable chemical industry: a review of achievements and prospects, *Green Chem.*, 6, 544, 2004.

3. Lynd, L.R., Wyman, C.E., and Gerngross, T.U., Biocommodity engineering, *Biotechnol. Prog.*, 15, 777, 1999.
4. Thiem, J., Applications of enzymes in synthetic carbohydrate-chemistry, *FEMS Microbiol. Rev.*, 16, 193, 1995.
5. Warwel, S. et al., Polymers and surfactants on the basis of renewable resources, *Chemosphere*, 43, 39, 2001.
6. Descotes, G. et al., Preparation of esters, ethers and acetals from unprotected sucrose, *Pol. J. Chem.*, 73, 1069, 1999.
7. Hill, K. and Rhode, O., Sugar-based surfactants for consumer products and technical applications, *Fett-Lipid*, 101, 25, 1999.
8. de Castro, H.F. et al., Modification of oils and fats by biotransformation, *Quim. Nova*, 27, 146, 2004.
9. Mulhaupt, R., The use of renewable resources: possibilities and limitations, *Chimia*, 50, 191, 1996.
10. Tundo, P. et al., Synthetic pathways and processes in green chemistry. Introductory overview, *Pure Appl. Chem.*, 72, 1207, 2000.
11. Holmberg, K., Natural surfactants, *Curr. Opin. Colloid Interface Sci.*, 6, 148, 2001.
12. Nakayama, M. et al., Studies on growth characteristics of *Bacillus* strains isolated from a liquid seasoning, *J. Japan. Soc. Food Sci. Technol.*, 50, 537, 2003.
13. Ferrer, M. et al., Synthesis of sugar esters in solvent mixtures by lipases from *Thermomyces lanuginosus* and *Candida antarctica* B, and their antimicrobial properties, *Enzyme Microb. Technol.*, 36, 391, 2005.
14. Okabe, S. et al., Disaccharide esters screened for inhibition of tumor necrosis factor- α release are new anti-cancer agents, *Jpn. J. Cancer Res.*, 90, 669, 1999.
15. Ferrer, M. et al., Antitumour activity of fatty acid maltotriose esters obtained by enzymatic synthesis, *Biotechnol. Appl. Biochem.*, 42, 35, 2005.
16. Devulapalle, K.S. et al., Effect of carbohydrate fatty acid esters on *Streptococcus sobrinus* and glucosyltransferase activity, *Carbohydr. Res.*, 339, 1029, 2004.
17. Michaud, J.P. and Mckenzie, C.L., Safety of a novel insecticide, sucrose octanoate, to beneficial insects in *Florida* citrus, *Fla. Entomol.*, 87, 6, 2004.
18. Ferrer, M. et al., Comparative surface activities of di- and trisaccharide fatty acid esters, *Langmuir*, 18, 667, 2002.
19. Queneau, Y., Fitremann, J., and Trombotto, S., The chemistry of unprotected sucrose: the selectivity issue, *C. R. Chim.*, 7, 177, 2004.
20. Cruces, M.A. et al., Improved synthesis of sucrose fatty acid monoesters, *J. Am. Oil Chem. Soc.*, 78, 541, 2001.
21. Plou, F.J. et al., Enzymatic synthesis of partially acylated sucroses, *Ann. N. Y. Acad. Sci.*, 750, 332, 1995.
22. Polat, T. and Linhardt, R.J., Syntheses and applications of sucrose-based esters, *J. Surf. Deterg.*, 4, 415, 2001.
23. Plou, F.J. et al., Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent, *J. Biotechnol.*, 96, 55, 2002.
24. Degn, P. et al., Lipase-catalysed synthesis of glucose fatty acid esters in tert-butanol, *Biotechnol. Lett.*, 21, 275, 1999.
25. Fregapane, G. et al., Enzymatic synthesis of monosaccharide fatty-acid esters and their comparison with conventional products, *J. Am. Oil Chem. Soc.*, 71, 87, 1994.
26. Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzyme-Catalysed Reactions at <http://www.chem.qmw.ac.uk/iubmb/enzyme>; <http://us.expasy.org/enzyme>, 2005.
27. Riva, S. et al., Protease-catalyzed regioselective esterification of sugars and related compounds in anhydrous dimethylformamide, *J. Am. Chem. Soc.*, 110, 584, 1988.
28. Polat, T., Bazin, H.G., and Linhardt, R.J., Enzyme catalyzed regioselective synthesis of sucrose fatty acid ester surfactants, *J. Carbohydr. Chem.*, 16, 1319, 1997.
29. Husband, F.A. et al., Comparison of foaming and interfacial properties of pure sucrose mono-laurates, dilaurate and commercial preparations, *Food Hydrocolloid.*, 12, 237, 1998.

30. Ferrer, M. et al., Lipase-catalyzed regioselective acylation of sucrose in two-solvent mixtures, *Biotechnol. Bioeng.*, 65, 10, 1999.
31. Kim, J.E. et al., Effect of salt hydrate pair on lipase-catalyzed regioselective monoacylation of sucrose, *Biotechnol. Bioeng.*, 57, 121, 1998.
32. Woudenberg van O.M., van Rantwijk, F., and Sheldon, R.A., Regioselective acylation of disaccharides in tert-butyl alcohol catalyzed by *Candida antarctica* lipase, *Biotechnol. Bioeng.*, 49, 328, 1996.
33. Rich, J.O., Bedell, B.A., and Dordick, J.S., Controlling enzyme-catalyzed regioselectivity in sugar ester synthesis, *Biotechnol. Bioeng.*, 45, 426, 1995.
34. Fuentes, G. et al., Computational studies of subtilisin-catalyzed transesterification of sucrose: importance of entropic effects, *Chembiochem*, 3, 907, 2002.
35. Park, O.J., Kim, D.Y., and Dordick, J.S., Enzyme-catalyzed synthesis of sugar-containing monomers and linear polymers, *Biotechnol. Bioeng.*, 70, 208, 2000.
36. Wang, X. et al., Chemo-enzymatic synthesis of disaccharide-branched copolymers with high molecular weight, *Carbohydr. Polym.*, 60, 357, 2005.
37. Pedersen, N.R. et al., Efficient transesterification of sucrose catalysed by the metalloprotease thermolysin in dimethylsulfoxide, *FEBS Lett.*, 519, 181, 2002.
38. Fuentes, G., Ballesteros, A., and Verma, C.S., Specificity in lipases: a computational study of transesterification of sucrose, *Protein Sci.*, 13, 3092, 2004.
39. Ferrer, M. et al., A simple procedure for the regioselective synthesis of fatty acid esters of maltose, leucrose, maltotriose and *N*-dodecyl maltosides, *Tetrahedron*, 56, 4053, 2000.
40. Raku, T. et al., Enzymatic synthesis of trehalose esters having lipophilicity, *J. Biotechnol.*, 100, 203, 2003.
41. Wu, Q. et al., Enzyme-catalyzed regioselective synthesis of novel raffinose vinyl esters, *Chem. J. Chin. Univ. Chin.*, 24, 1806, 2003.
42. Steverink de Zoete, M.C. et al., Enzymatic synthesis and NMR studies of acylated sucrose acetates, *Green Chem.*, 1, 153, 1999.
43. Carrea, G. et al., Enzymatic synthesis of various 1'-O-sucrose and 1-O-fructose esters, *J. Chem. Soc. Perkin Trans. I*, 1057, 1989.
44. Jhurry, D. et al., Sucrose-based polymers. 1. Linear-polymers with sucrose side-chains, *Macromol. Chem. Phys.*, 193, 2997, 1992.
45. Chen, K.Q. and Arnold, F.H., Tuning the activity of an enzyme for unusual environments—sequential random mutagenesis of subtilisin-E for catalysis in dimethylformamide, *Proc. Natl. Acad. Sci. USA*, 90, 5618, 1993.
46. Plou, F.J. et al., Acylation of sucrose with vinyl esters using immobilized hydrolases: demonstration that chemical catalysis may interfere with enzymatic catalysis, *Biotechnol. Lett.*, 21, 635, 1999.
47. Chamouleau, F. et al., Influence of water activity and water content on sugar esters lipase-catalyzed synthesis in organic media, *J. Mol. Catal., B Enzym.*, 11, 949, 2001.
48. Arcos, J.A., Hill, C.G., and Otero, C., Kinetics of the lipase-catalyzed synthesis of glucose esters in acetone, *Biotechnol. Bioeng.*, 73, 104, 2001.
49. Pedersen, N.R. et al., Effect of fatty acid chain length on initial reaction rates and regioselectivity of lipase-catalysed esterification of disaccharides, *Carbohydr. Res.*, 337, 1179, 2002.
50. Simerska, P. et al., Regioselective enzymatic acylation of *N*-acetylhexosamines, *J. Mol. Catal., B Enzym.*, 29, 219, 2004.
51. Castillo, E. et al., Lipase-catalyzed synthesis of xylitol monoesters: solvent engineering approach, *J. Biotechnol.*, 102, 251, 2003.
52. Reyes-Duarte, D. et al., Parameters affecting productivity in the lipase-catalysed synthesis of sucrose palmitate, *Biocatal. Biotransform.*, 23, 19, 2005.
53. Flores, M.V. and Halling, P.J., Full model for reversible kinetics of lipase-catalyzed sugar-ester synthesis in 2-methyl 2-butanol, *Biotechnol. Bioeng.*, 78, 794, 2002.
54. Flores, M.V. et al., Influence of glucose solubility and dissolution rate on the kinetics of lipase catalyzed synthesis of glucose laurate in 2-methyl 2-butanol, *Biotechnol. Bioeng.*, 78, 814, 2002.
55. Rendon, X., Lopez-Munguia, A., and Castillo, E., Solvent engineering applied to lipase-catalyzed glycerolysis of triolein, *J. Am. Oil Chem. Soc.*, 78, 1061, 2001.

56. Ferrer, M. et al., Effect of the immobilization method of lipase from *Thermomyces lanuginosus* on sucrose acylation, *Biocatal. Biotransform.*, 20, 63, 2002.
57. Voutsas, E.C. et al., Solubility measurements of fatty acid glucose and sucrose esters in 2-methyl-2-butanol and mixtures of 2-methyl-2-butanol with dimethyl sulfoxide, *J. Chem. Eng. Data*, 47, 1517, 2002.
58. Plou, F.J., Ferrer, M., and Ballesteros, A., Transesterification-Biological, in *Encyclopedia of Catalysis*, Vol. 6, Horváth, I.T., Ed., Wiley-Interscience, New York, 2003, p. 483.
59. Pulido, R. and Gotor, V., Towards the selective acylation of secondary hydroxyl-groups of carbohydrates using oxime esters in an enzyme-catalyzed process, *Carbohydr. Res.*, 252, 55, 1994.
60. Wang, Y.F. et al., Lipase-catalyzed irreversible transesterifications using enol esters as acylating reagents—preparative enantioselective and regioselective syntheses of alcohols, glycerol derivatives, sugars, and organometallics, *J. Am. Chem. Soc.*, 110, 7200, 1988.
61. Weber, H.K., Stecher, H., and Faber, K., Sensitivity of microbial lipases to acetaldehyde formed by acyl-transfer reactions from vinyl esters, *Biotechnol. Lett.*, 17, 803, 1995.
62. Weber, H.K. and Faber, K., Stabilization of lipases against deactivation by acetaldehyde formed in acyl transfer reactions, *Meth. Enzymol.*, 286, 509, 1997.
63. Eggleston, G. and Cote, G.L., Oligosaccharides in food and agriculture, in *Oligosaccharides in Food and Agriculture*, Eggleston, G. and Cote, G.L., Eds., American Chemical Society, Washington, 2003, chap. 1.
64. McNaught, A.D., International union of pure and applied chemistry and international union of biochemistry and molecular biology—joint commission on biochemical nomenclature—nomenclature of carbohydrates (Recommendations 1996) (Reprinted from *Pure Appl. Chem.*, 68, 1919–2008, 1996), 43, 1997.
65. Macmillan, D. and Daines, A.M., Recent developments in the synthesis and discovery of oligosaccharides and glycoconjugates for the treatment of disease, *Curr. Med. Chem.*, 10, 2733, 2003.
66. Kren, V. and Thiem, J., Glycosylation employing bio-systems: from enzymes to whole cells, *Chem. Soc. Rev.*, 26, 463, 1997.
67. Ichikawa, Y. et al., Synthesis of oligosaccharides using glycosyltransferases, *J. Synth. Org. Chem. Jpn.*, 50, 441, 1992.
68. Coutinho, P.M. and Henrissat, B., Carbohydrate-Active Enzymes Server at URL: <http://afmb.cnrs-mrs.fr/CAZY/>, 1999.
69. Planas, A. and Faijes, M., Glycosidases and glycosynthases in enzymatic synthesis of oligosaccharides. An overview, *Afinidad*, 59, 295, 2002.
70. Ajisaka, K. and Yamamoto, Y., Control of the regioselectivity in the enzymatic syntheses of oligosaccharides using glycosidases, *Trends Glycosci. Glycotechnol.*, 14, 1, 2002.
71. Plou, F.J. et al., Glucosyltransferases acting on starch or sucrose for the synthesis of oligosaccharides, *Can. J. Chem.*, 80, 743, 2002.
72. Crout, D.H. and Vic, G., Glycosidases and glycosyl transferases in glycoside and oligosaccharide synthesis, *Curr. Opin. Chem. Biol.*, 2, 98, 1998.
73. Davies, G.J., Charnock, S.J., and Henrissat, B., The enzymatic synthesis of glycosidic bonds: “glycosynthases” and glycosyltransferases, *Trends Glycosci. Glycotechnol.*, 13, 105, 2001.
74. Mackenzie, L.F. et al., Glycosynthases: mutant glycosidases for oligosaccharide synthesis, *J. Am. Chem. Soc.*, 120, 5583, 1998.
75. Malet, C. and Planas, A., From beta-glucanase to beta-glucansynthase: glycosyl transfer to alpha-glycosyl fluorides catalyzed by a mutant endoglucanase lacking its catalytic nucleophile, *FEBS Lett.*, 440, 208, 1998.
76. Perugino, G. et al., Recent advances in the oligosaccharide synthesis promoted by catalytically engineered glycosidases, *Adv. Synth. Catal.*, 347, 941, 2005.
77. Tungland, B.C., Fructooligosaccharides and other fructans: Structures and occurrence, production, regulatory aspects, food applications, and nutritional health significance, in *Oligosaccharides in Food and Agriculture*, Eggleston, G. and Cote, G.L., Eds., American Chemical Society, Washington, 2003, p. 135.
78. Monsan, P. and Paul, F., Enzymatic synthesis of oligosaccharides, *FEMS Microbiol. Rev.*, 16, 187, 1995.

79. Bornet, F.R.J. et al., Nutritional aspects of short-chain fructooligosaccharides: natural occurrence, chemistry, physiology and health implications, *Dig. Liver Dis.*, 34, S111, 2002.
80. Probert, H.M. and Gibson, G.R., Investigating the prebiotic and gas-generating effects of selected carbohydrates on the human colonic microflora, *Lett. Appl. Microbiol.*, 35, 473, 2002.
81. Fooks, L.J., Fuller, R., and Gibson, G.R., Prebiotics, probiotics and human gut microbiology, *Int. Dairy J.*, 9, 53, 1999.
82. Roberfroid, M., Functional food concept and its application to prebiotics, *Dig. Liver Dis.*, 34, S105, 2002.
83. Gibson, G.R. and Ottaway, R.A., *Prebiotics: New Developments in Functional Foods*, Chandos Publishing (Oxford), Oxford, 2000, chap. 2.
84. Tuohy, K.M. et al., Modulation of the human gut microflora towards improved health using prebiotics—assessment of efficacy, *Curr. Pharm. Design.*, 11, 75, 2005.
85. Grizard, D. and Barthomeuf, C., Non-digestible oligosaccharides used as prebiotic agents: mode of production and beneficial effects on animal and human health, *Reprod. Nutr. Dev.*, 39, 563, 1999.
86. Antosova, M. and Polakovic, M., Fructosyltransferases: the enzymes catalyzing production of fructooligosaccharides, *Chem. Pap. Chem. Zvesti*, 55, 350, 2001.
87. Hirayama, M., Sumi, N., and Hidaka, H., Purification and properties of a fructooligosaccharide-producing beta-fructofuranosidase from *Aspergillus niger* ATCC-20611, *Agric. Biol. Chem.*, 53, 667, 1989.
88. Sangeetha, P.T., Ramesh, M.N., and Prapulla, S.G., Fructooligosaccharide production using fructosyl transferase obtained from recycling culture of *Aspergillus oryzae* CFR 202, *Process Biochem.*, 40, 1085, 2005.
89. Shin, H.T. et al., Production of fructo-oligosaccharides from molasses by *Aureobasidium pullulans* cells, *Bioresour. Technol.*, 93, 59, 2004.
90. Fernandez, R.C. et al., Production of fructooligosaccharides by beta-fructofuranosidase from *Aspergillus* sp. 27H, *J. Chem. Technol. Biotechnol.*, 79, 268, 2004.
91. Olivares-Illana, V. et al., Characterization of a cell-associated inulosucrase from a novel source: a *Leuconostoc citreum* strain isolated from Pozol, a fermented corn beverage of Mayan origin, *J. Ind. Microbiol. Biotechnol.*, 28, 112, 2002.
92. Velasco, J. and Adrio, J.L., Microbial enzymes for food-grade oligosaccharide biosynthesis, in *Microorganisms for Health Care, Food and Enzyme Production*, Barredo, J.L., Ed., Research Signpost, Trivandrum, Kerala, India, 2002.
93. Alberto, F. et al., The three-dimensional structure of invertase (beta-fructosidase) from *Thermotoga maritima* reveals a bimodular arrangement and an evolutionary relationship between retaining and inverting glycosidases, *J. Biol. Chem.*, 279, 18903, 2004.
94. Nguyen, Q.D. et al., Purification and some properties of beta-fructofuranosidase from *Aspergillus niger* IMI303386, *Process Biochem.*, 40, 2461, 2005.
95. Buchholz, K., Kasche, V., and Bornscheuer, U.T., *Biocatalysts and Enzyme Technology*, Wiley-VCH, Weinheim, Germany, 2005, chap. 2.
96. Ghazi, I. et al., Immobilisation of fructosyltransferase from *Aspergillus aculeatus* on epoxy-activated Sepabeads EC for the synthesis of fructo-oligosaccharides, *J. Mol. Catal., B Enzym.*, 35, 19, 2005.
97. Chiang, C.J. et al., Immobilization of beta-fructofuranosidases from *Aspergillus* on methacrylamide-based polymeric beads for production of fructooligosaccharides, *Biotechnol. Prog.*, 13, 577, 1997.
98. Tanriseven, A. and Aslan, Y., Immobilization of Pectinex Ultra SP-L to produce fructooligosaccharides, *Enzyme Microb. Technol.*, 36, 550, 2005.
99. Euzenat, O., Guibert, A., and Combes, D., Production of fructo-oligosaccharides by levansucrase from *Bacillus subtilis* C4, *Process Biochem.*, 32, 237, 1997.
100. Tambara, Y. et al., Structural analysis and optimised production of fructo-oligosaccharides by levansucrase from *Acetobacter diazotrophicus* SRT4, *Biotechnol. Lett.*, 21, 117, 1999.
101. Trujillo, L.E. et al., Fructo-oligosaccharides production by the *Gluconacetobacter diazotrophicus* levansucrase expressed in the methylotrophic yeast *Pichia pastoris*, *Enzyme Microb. Technol.*, 28, 139, 2001.
102. Kim, M.G. et al., Synthesis of methyl beta-D-fructoside catalyzed by levansucrase from *Rahnella aquatilis*, *Enzyme Microb. Technol.*, 27, 646, 2000.

103. Gonzalez-Muñoz, F. et al., Enzymatic synthesis of fructosyl glycerol, *J. Carbohydr. Chem.*, 18, 275, 1999.
104. Park, H.E. et al., Enzymatic synthesis of fructosyl oligosaccharides by levansucrase from *Microbacterium laevaniformans* ATCC 15953, *Enzyme Microb. Technol.*, 32, 820, 2003.
105. Meng, G.Y. and Fütterer, K., Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase, *Nat. Struct. Biol.*, 10, 935, 2003.
106. Ozimek, L.K. et al., Site-directed mutagenesis study of the three catalytic residues of the fructosyl-transferases of *Lactobacillus reuteri* 121, *FEBS Lett.*, 560, 131, 2004.
107. Grizard, D. and Barthomeuf, C., Enzymatic synthesis and structure determination of NEO-FOS, *Food Biotechnol.*, 13, 93, 1999.
108. Kilian, S.G. et al., Transport-limited sucrose utilization and neokestose production by *Phaffia rhodozyma*, *Biotechnol. Lett.*, 18, 975, 1996.
109. Kritzinger, S.M. et al., The effect of production parameters on the synthesis of the prebiotic trisaccharide, neokestose, by *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*), *Enzyme Microb. Technol.*, 32, 728, 2003.
110. Hayashi, S. et al., Production of a novel syrup containing neofructo-oligosaccharides by the cells of *Penicillium citrinum*, *Biotechnol. Lett.*, 22, 1465, 2000.
111. Kilian, S. et al., The effects of the novel bifidogenic trisaccharide, neokestose, on the human colonic microbiota, *World J. Microbiol. Biotechnol.*, 18, 637, 2002.
112. Marx, S.P., Winkler, S., and Hartmeier, W., Metabolization of beta-(2,6)-linked fructose-oligosaccharides by different bifidobacteria, *FEMS Microbiol. Lett.*, 182, 163, 2000.
113. Bekers, M. et al., Fructooligosaccharide and levan producing activity of *Zymomonas mobilis* extracellular levansucrase, *Process Biochem.*, 38, 701, 2002.
114. Devulapalle, K.S. and Mooser, G., Glucosyltransferase inactivation reduces dental caries, *J. Dent. Res.*, 80, 466, 2001.
115. Mooser, G. and Iwaoka, K.R., Sucrose 6- α -D-glucosyltransferase from *Streptococcus sobrinus*—characterization of a glucosyl-enzyme complex, *Biochemistry*, 28, 443, 1989.
116. Monchois, V., Willemot, R.M., and Monsan, P., Glucansucrases: mechanism of action and structure-function relationships, *FEMS Microbiol. Rev.*, 23, 131, 1999.
117. Robyt, J.F. and Walseth, T.F., Mechanism of dextranucrase action. 4. Mechanism of acceptor reactions of *Leuconostoc mesenteroides* B-512F dextranucrase, *Carbohydr. Res.*, 61, 433, 1978.
118. Robyt, J.F., Mechanism and action of glucansucrases, in *Enzymes for Carbohydrate Engineering*, Park, K.H., Robyt, J.F., and Choi, Y.D., Eds., Elsevier Science, Amsterdam, 1996, p. 1.
119. Jeanes, A. et al., Characterization and classification of dextrans from ninety-six strains of bacteria, *J. Am. Chem. Soc.*, 76, 5041, 1954.
120. Robyt, J.F. and Eklund, S.H., Relative, quantitative effects of acceptors in the reaction of *Leuconostoc mesenteroides* B-512F dextranucrase, *Carbohydr. Res.*, 121, 279, 1983.
121. Dols-Lafargue, M. et al., Factors affecting α -1,2 glucooligosaccharide synthesis by *Leuconostoc mesenteroides* NRRL B-1299 dextranucrase, *Biotechnol. Bioeng.*, 74, 498, 2001.
122. Gómez de Segura, A. et al., Encapsulation in LentiKats of dextranucrase from *Leuconostoc mesenteroides* NRRL B-1299, and its effect on product selectivity, *Biocatal. Biotransform.*, 21, 325, 2003.
123. Gómez de Segura, A. et al., Modulating the synthesis of dextran with the acceptor reaction using native and encapsulated dextranucrases, *Food Technol. Biotechnol.*, 42, 337, 2004.
124. Buchholz, K., Noll-Borchers, M., and Schwengers, D., Production of leucrose by dextranucrase, *Starch-Starke*, 50, 164, 1998.
125. Goulas, A.K. et al., Synthesis of isomaltooligosaccharides and oligodextrans in a recycle membrane bioreactor by the combined use of dextranucrase and dextranase, *Biotechnol. Bioeng.*, 88, 778, 2004.
126. Buchholz, K. and Seibel, J., Isomaltooligosaccharides, in *Oligosaccharides in Food and Agriculture*, Eggleston, G. and Coté, G.L., Eds., American Chemical Society, Washington, 2003, p. 63.
127. Djouzi, Z. et al., Degradation and fermentation of α -gluco-oligosaccharides by bacterial strains from human colon: *in vitro* and *in vivo* studies in gnotobiotic rats, *J. Appl. Bacteriol.*, 79, 117, 1995.

128. Simmering, R. and Blaut, M., Pro- and prebiotics—the tasty guardian angels? *Appl. Microbiol. Biotechnol.*, 55, 19, 2001.
129. Boucher, J. et al., Effect of non-digestible gluco-oligosaccharides on glucose sensitivity in high fat diet fed mice, *J. Physiol. Biochem.*, 59, 169, 2003.
130. Dols, M. et al., Structural characterization of the maltose acceptor-products synthesized by *Leuconostoc mesenteroides* NRRL B-1299 dextranucrase, *Carbohydr. Res.*, 305, 549, 1998.
131. Yamauchi, F. and Ohwada, Y., Synthesis of oligosaccharides by growing culture of *Leuconostoc mesenteroides*. 4. Oligosaccharide formation in presence of various types of glucobioses as acceptors, *Agric. Biol. Chem.*, 33, 1295, 1969.
132. Heincke, K. et al., Glucosylation by dextranucrase—modeling of reaction kinetics and unconventional products, *Ann. N. Y. Acad. Sci.*, 864, 203, 1998.
133. Qi, Q.S. and Zimmermann, W., Cyclodextrin glucanotransferase: from gene to applications, *Appl. Microbiol. Biotechnol.*, 66, 475, 2005.
134. Tonkova, A., Bacterial cyclodextrin glucanotransferase, *Enzyme Microb. Technol.*, 22, 678, 1998.
135. Alcalde, M. et al., Effect of chemical modification of cyclodextrin glycosyltransferase (CGTase) from *Thermoanaerobacter* sp. on its activity and product selectivity, *Ann. N. Y. Acad. Sci.*, 864, 183, 1998.
136. Zamost, B.L., Nielsen, H.K., and Starnes, R.L., Thermostable enzymes for industrial applications, *J. Ind. Microbiol.*, 8, 71, 1991.
137. van der Veen, B.A. et al., The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms, *Eur. J. Biochem.*, 267, 658, 2000.
138. Leemhuis, H. and Dijkhuizen, L., Engineering of hydrolysis reaction specificity in the transglycosylase cyclodextrin glycosyltransferase, *Biocatal. Biotransform.*, 21, 261, 2003.
139. Leemhuis, H. et al., Engineering cyclodextrin glycosyltransferase into a starch hydrolase with a high exo-specificity, *J. Biotechnol.*, 103, 203, 2003.
140. Leemhuis, H. et al., Conversion of cyclodextrin glycosyltransferase into a starch hydrolase by directed evolution: the role of alanine 230 in acceptor subsite+1, *Biochemistry*, 42, 7518, 2003.
141. Alcalde, M. et al., Succinylation of cyclodextrin glycosyltransferase from *Thermoanaerobacter* sp. 501 enhances its transferase activity using starch as donor, *J. Biotechnol.*, 86, 71, 2001.
142. Alcalde, M. et al., Chemical modification of lysine side chains of cyclodextrin glycosyltransferase from *Thermoanaerobacter* causes a shift from cyclodextrin glycosyltransferase to alpha-amylase specificity, *FEBS Lett.*, 445, 333, 1999.
143. Park, D.C., Kim, T.K., and Lee, Y.H., Characteristics of transglycosylation reaction of cyclodextrin glucanotransferase in the heterogeneous enzyme reaction system using extrusion starch as a glucosyl donor, *Enzyme Microb. Technol.*, 22, 217, 1998.
144. Nakamura, A., Haga, K., and Yamane, K., The transglycosylation reaction of cyclodextrin glucanotransferase is operated by a ping-pong mechanism, *FEBS Lett.*, 337, 66, 1994.
145. Martin, M.T. et al., Synthesis of malto-oligosaccharides via the acceptor reaction catalyzed by cyclodextrin glycosyltransferases, *Biocatal. Biotransform.*, 19, 21, 2001.
146. Aga, H. et al., Synthesis of 2-O-alpha-D-glucopyranosyl L-ascorbic-acid by cyclomaltodextrin glucanotransferase from *Bacillus stearothermophilus*, *Agric. Biol. Chem.*, 55, 1751, 1991.
147. Kim, T.K., Park, D.C., and Lee, Y.H., Synthesis of glucosyl-sugar alcohols using glycosyltransferases and structural identification of glucosyl-maltitol, *J. Microbiol. Biotechnol.*, 7, 310, 1997.
148. Ohara, S. and Hishiyama, S., Utilization of triterpenoids. 1. Synthesis of betulin glycosides by cyclodextrin glycosyltransferase, *Mokuzai Gakkaishi*, 40, 444, 1994.
149. Kometani, T. et al., Transglycosylation to hesperidin by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species in alkaline pH and properties of hesperidin glycosides, *Biosci. Biotechnol. Biochem.*, 58, 1990, 1994.
150. Kometani, T. et al., Synthesis of hesperidin glycosides by cyclodextrin glucanotransferase and stabilization of the natural pigments, *J. Jpn. Soc. Food Sci. Technol.*, 42, 376, 1995.
151. Robyt, J.F., Mechanisms in the glucanucrase synthesis of polysaccharides and oligosaccharides from sucrose, *Adv. Carbohydr. Chem. Biochem.*, 51, 133, 1995.
152. Canedo, M. et al., Production of maltosylfructose (erlose) with levansucrase from *Bacillus subtilis*, *Biocatal. Biotransform.*, 16, 475, 1999.

153. Baciú, L.E. et al., Investigations of the transfructosylation reaction by fructosyltransferase from *B. subtilis* NCIMB 11871 for the synthesis of the sucrose analogue galactosyl-fructoside, *J. Biotechnol.*, 116, 347, 2005.
154. Sato, M. et al., Synthesis of novel sugars, oligoglucosyl-inositols, and their growth stimulating effect for *Bifidobacterium*, *Biotechnol. Lett.*, 13, 69, 1991.
155. Demuth, K., Jordening, H.J., and Buchholz, K., Oligosaccharide synthesis by dextransucrase: new unconventional acceptors, *Carbohydr. Res.*, 337, 1811, 2002.
156. Tachibana, Y. et al., Purification and characterization of an extremely thermostable cyclomalto-dextrin glucanotransferase from a newly isolated hyperthermophilic archaeon, a *Thermococcus* sp., *Appl. Environ. Microbiol.*, 65, 1991, 1999.
157. Jun, H.K., Bae, K.M., and Kim, S.K., Production of 2-*O*- α -D -glucopyranosyl L-ascorbic acid using cyclodextrin glucanotransferase from *Paenibacillus* sp., *Biotechnol. Lett.*, 23, 1793, 2001.
158. Nakano, H. et al., Syntheses of glucosides with trimethylolpropane and 2 related polyol moieties by cyclodextrin glucanotransferase and their esterification by lipase, *J. Ferment. Bioeng.*, 73, 237, 1992.
159. Kometani, T. et al., Synthesis of neohesperidin glycosides and naringin glycosides by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species, *Biosci. Biotechnol. Biochem.*, 60, 645, 1996.
160. Funayama, M. et al., Enzymatic-synthesis of (+)catechin- α -glucoside and its effect on tyrosinase activity, *Biosci. Biotechnol. Biochem.*, 57, 1666, 1993.

18 Efficient Methods and Instrumentation for Engineering Custom Enzymes

Steven J. Robles, William J. Coleman, and Mary M. Yang

CONTENTS

18.1	Nature's Diversity	489
18.2	Directed Evolution: Reversing the Metaphor of the Genetic Algorithm	490
18.3	The Reality of the Screening Bottleneck	492
18.4	Solid-Phase Enzyme Screening Technology: Maximizing Density for High Throughput	493
18.5	Synthesis of Pharmaceutical Intermediates: Nitrilase	495
18.6	Modification of Biopolymers: Cellulase and Other Carbohydrate Enzymes	496
18.7	Future Prospects	499
18.7.1	Advantages of Customized Enzymes	499
18.7.2	Green Chemistry for Cheaper, Cleaner Pharmaceutical Manufacturing	499
18.7.3	Value of Modified Polymers	499
	References	499

18.1 NATURE'S DIVERSITY

The quest to identify highly active and stable biocatalysts generally requires sampling a highly diverse pool of enzyme variants in order to retrieve rare mutants with desirable properties. One major source of “natural” diversity is contained in the genomes of environmental microorganisms, the vast majority of which have never been cultivated or characterized [1,2]. Methods for screening such libraries for a number of different enzyme activities have been described [3]. However, despite the advantages of screening naturally occurring enzymes, the properties of these biocatalysts are often not optimal for industrial chemistry, as these processes typically involve reaction conditions that are rarely or never encountered in the natural environment [4]. In addition, the intrinsic properties of the enzyme (e.g., substrate affinity, catalytic efficiency, stability) may not be appropriate for the desired substrate. Properties that typically require optimization include:

- Regio- and enantioselectivity
- Activity on a novel substrate
- Resistance to inhibitors, extreme pH, or organic solvents
- Thermostability
- Thermoactivity

Methods have been developed to overcome the natural limitations of enzymes by using *in vitro* mutagenesis to create artificial diversity. Most of these methods are actually adapted from natural evolutionary processes. Several of these directed evolution methods are related to genetic algorithms (GAs), and will be briefly described in Section 18.2.

18.2 DIRECTED EVOLUTION: REVERSING THE METAPHOR OF THE GENETIC ALGORITHM

GAs were conceived by computer scientists several decades ago as a search technique to quickly find solutions to combinatorial optimization problems by mimicking the biological processes of mutation and recombination [5]. In this metaphor on biological evolution, GAs equate biological chromosomes with digital strings of alphanumeric characters. These strings exist within populations, and each can be scored for fitness. Thus, the basic elements of a digital computer's GA are as follows:

1. Population of strings
2. Random mutation along each string
3. Random recombination among the strings
4. Scoring mechanism for fitness
5. Method of recursion

This metaphor was “reversed” in the early 1990s by molecular biologists who began using GAs to engineer proteins in an effort to solve the “protein folding problem,” wherein protein structure and function are calculated from the amino acid sequence. These initial studies were performed by computer simulations [6–8], followed by *in vitro* evolution of protein genes in the laboratory [9–12].

In the laboratory, each of the five elements of a digital GA were reembodyed in biological material and procedures:

1. Population of DNA molecules
2. Mutation (e.g., using an error-prone polymerase chain reaction [PCR] or a degenerate cassette)
3. Recombination (production of DNA strands having random combinations of mutations)
4. Selection or screening criteria based on the properties of the encoded protein
5. Propagating “fit” clones

In what later became known as sequential random mutagenesis (SRM) for protein evolution, step 3 was not employed. This noncanonical GA utilized error-prone PCR mutagenesis to create a pool of point mutations whose phenotypes could be screened, resulting in the isolation of improved variants after several iterations [13]. However, methods based on introducing point mutations without thorough recombination require a large amount of screening because many of the point mutations will be either silent or deleterious, and beneficial combinations of mutations are unlikely to occur within the same DNA strand [14,15].

DNA shuffling [11,12] and recursive ensemble mutagenesis (REM) [9,10] are canonical GAs, using both mutation and random recombination as part of all five steps listed above. REM is based on combinatorial cassette mutagenesis and predates DNA shuffling. A subtle but important difference between the two methods is that the recombination operator in

DNA shuffling is only useful if the point mutations found in seminal strings are independently favorable. This requirement limits its ability to solve hard combinatorial problems where simultaneous mutations are necessary. However, as DNA shuffling uses PCR to amplify families of gene fragments and thereby reconstitute chimeric genes containing various combinations of point mutations, the advantage is that beneficial mutations scattered throughout various DNA fragments can be brought together into the same construct so that their cumulative and synergistic effects can be evaluated [14]. Likewise, deleterious mutations can be eliminated. The disadvantages of PCR-based reassembly are that (1) proper annealing of the fragments requires significant sequence similarity, and recombination is strongly favored in regions of high homology [16,17]; (2) truncated and multimeric sequences are generated by the mutagenesis procedure [18]; and (3) potentially desirable point mutations on different fragments cannot be located too close together in the sequence because their rate of recombination will be low.

The REM procedure (Figure 18.1) seeks to find a set of “optimal” amino acids at preselected positions in a given protein so that its functional behavior can be enhanced or appropriately altered. REM exploits the relationship between genotype (DNA) and phenotype (protein) by incorporating a computer program called CyberDope. This is an interactive program that restricts the sequence complexity of mixed bases in the synthesis of DNA oligonucleotides such that the combinatorial complexity can be reduced. This is accomplished by favoring functional and/or unique mutations in the library. The program calculates

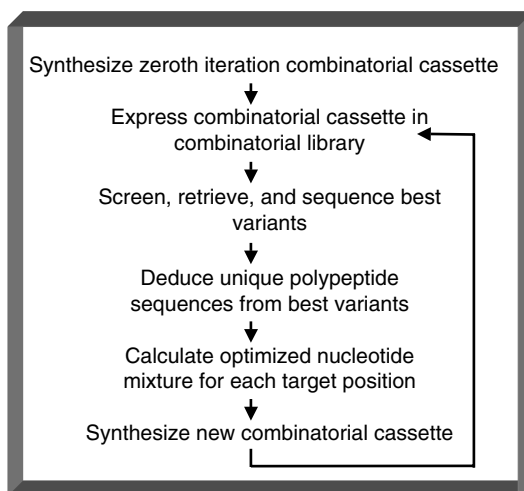


FIGURE 18.1 Flowchart for recursive ensemble mutagenesis (REM), which involves the iterative use of combinatorial cassette mutagenesis. Typical steps in the process are shown here. For the zeroth iteration, a combinatorial cassette can be synthesized using phylogenetic data, randomized codons [NN(G,C) or NNN], sequence information from prior screening (e.g., from error-prone polymerase chain reaction [PCR]), or elements of rational design. The starting cassette is expressed in a library, which is then screened for functional or positive clones. The positive clones are sequenced, and the unique polypeptide sequences are identified. These unique sequences form a target set of amino acids at a particular residue. Based on the target set at each position, the CyberDope program calculates a nucleotide mixture biased toward amino acids in the target set. The output of the program is used to synthesize a new cassette, which is incorporated into another round of mutagenesis and screening. A number of algorithms for modifying or refining this process are included in the CyberDope program. Constraints based on amino acid physicochemical properties can also be built into the design of the combinatorial cassette. Therefore, the program provides improved control over the population diversity.

optimized nucleotide mixtures using algorithms to maximize the occurrence of a specified set of amino acid residues. Information from phylogeny, PCR mutagenesis, or other previous rounds of combinatorial cassette mutagenesis can be used in an iterative manner to search sequence space more efficiently. Multiple REM segments can also be combined to potentially mutagenize entire proteins. With a 16-site library, a 10 million-fold enrichment of functional and unique mutants, compared to random cassette mutagenesis, has been achieved [10].

REM is especially advantageous for targeting particular regions of the protein sequence, such as an antibody-binding site or a motif that comprises a portion of the enzyme active site [19]. This kind of targeted mutagenesis may be more advantageous than previously realized. For cases in which the desired improvement involves enantioselectivity, substrate selectivity, or new catalytic activity, an analysis of directed evolution experiments by Morley and Kazlauskas [20] suggests that mutations close to the enzyme active site have a higher probability of generating enhanced phenotypes than do more distant mutations.

REM may be seen as a GA constrained by experimental requirements or physicochemical properties. The repertoire of substitutions can be restricted to a certain class—for example, charged or aromatic amino acids. Nucleotide mixtures can also be determined using other physicochemical criteria to change the average molar volume, hydropathy, pK_a , or other parameters known to affect protein structure and function. These constraints give rise to a powerful new optimization and diversification component not found in traditional GAs.

18.3 THE REALITY OF THE SCREENING BOTTLENECK

Although the GA-based mutagenesis techniques described in [Section 18.2](#) make it possible to explore sequence space with greater thoroughness and efficiency, finding a truly novel protein or enzyme can still be fundamentally a numbers game. For example, a relatively small protein (e.g., length = 100) may be represented by 20^{100} possible sequences. Randomly incorporating all 20 amino acids at just six different positions in a given protein will produce a theoretical complexity of 64 million unique sequences. The need to screen large libraries is also due in part to the scarcity of positive phenotypes. Thus, the demands placed on the available activity-based screening procedures to process such large libraries efficiently and accurately has created the “screening bottleneck.”

To deal with this challenge, various liquid-phase platforms have been used to screen libraries at high throughput [21]. Commercial microplate systems employ 96-, 384-, or 1536-well plates with individual reaction volumes ranging from about 10 to 200 μ L. The advantage of using these systems is that they are versatile and adaptable. However, due to the limited well density, screening a somewhat modest library of 1 million variants requires between 700 and 10,000 plates, with a total substrate volume of 10 to 200 L. The investment required in terms of robotics, reagents, and laboratory space for handling, analyzing, and washing large numbers of microplates can therefore be substantial. This means that most experiments utilize a total throughput of fewer than 10,000 variants.

Fluorescence-activated cell sorting (FACS) has also been used to screen microbial cells expressing enzymes [22], and this has been extended to mutagenized enzyme libraries [23]. The chief advantage of FACS is its extremely high throughput, which is typically 10,000 to 50,000 cells/s. However, the applicability of the method is limited by several factors: (1) the product to be detected must be fluorescent; (2) the substrate must freely enter the cell; (3) the product must not leak out of the cell; (4) only a single cell can be measured at a time; and (5) only end-point assays can be performed. The penetration and/or leakage problems can be resolved by using cell-surface display of the enzyme and trapping the products [23], or by encapsulating the cells in microdroplets [24].

However, despite their proven utility for screening many types of enzymes, liquid-phase techniques are not the only assay format that can be applied to the problem of the screening bottleneck. In order to pursue the goals of greater efficiency, versatility, information content, and cost savings (without sacrificing accuracy or performance), a platform of solid-phase methods, software, and instrumentation known commercially as Kcat technology was therefore developed [25]. This technology is designed to reduce or eliminate some of the drawbacks inherent in conventional liquid-phase screening. By providing very high throughput, the problem of the screening bottleneck can be reduced. Assay design can be more flexible, and sample handling can also be vastly simplified. Application of this method to enzyme engineering will be described in the following sections.

18.4 SOLID-PHASE ENZYME SCREENING TECHNOLOGY: MAXIMIZING DENSITY FOR HIGH THROUGHPUT

A key objective of solid-phase screening is to achieve very high throughput by maximizing the cell density. This can be accomplished in a straightforward manner by using microcolonies randomly distributed on a microporous surface. The basic solid-phase assay for high-throughput enzyme screening is shown in Figure 18.2. Typically, the enzyme gene is expressed using a plasmid vector in a microbial host, such as *Escherichia coli*. The transformed cells containing the mutagenized plasmid library are deposited randomly on an assay disk, which consists of a very thin, microporous membrane. The following steps are performed:

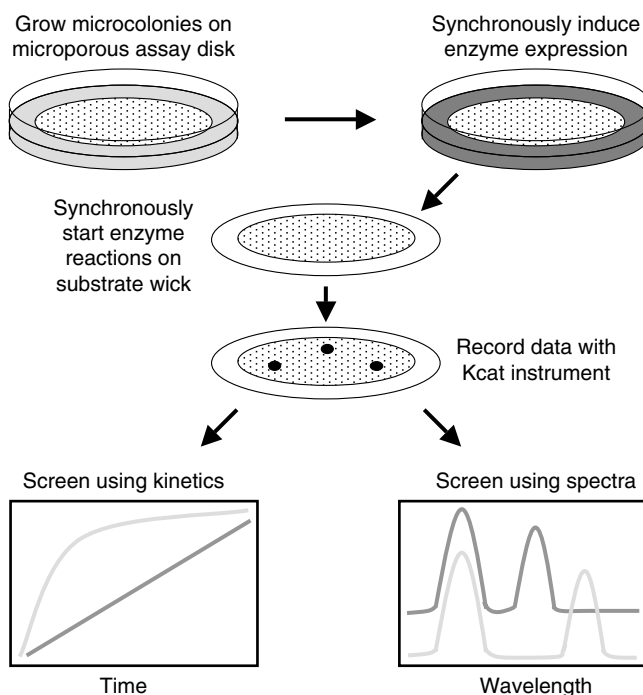


FIGURE 18.2 Solid-phase screening flowchart. The basic steps involved in preparing and analyzing an assay disk using Kcat technology are shown. The kinetics mode can be used to identify variants with enhanced activity, while the spectral mode can be used to compare activity on different substrates or detect novel colored products.

1. Place assay disk with transformed cells on nutrient agar containing the appropriate antibiotics, and incubate the disk until microcolonies develop on the surface.
2. (Optional) Transfer the assay disk to nutrient agar containing an inducer to synchronously start expression of the enzyme in all of the microcolonies.
3. Transfer the assay disk to a “wick” impregnated with substrate inside the Kcat instrument so that the enzyme reactions are synchronously initiated in all of the microcolonies.
4. Collect data with the Kcat instrumentation as a function of wavelength or time.
5. Perform massively parallel data analysis and sorting using spectral and/or time-based information.

Enzymatic activity can be detected by using chromogenic or fluorogenic substrates, colorimetric indicators, or coupled enzyme reactions. The Kcat instrument acquires images of the assay disk over time at the appropriate wavelength(s) to determine the enzymatic activities of the microcolonies on the disk. The data obtained consist of full spectra and kinetics for every pixel in the image. Kcat software analyzes the disk to identify the desired microcolonies, and then the “positive” plasmids are retrieved and purified.

Analysis is performed using software that processes and classifies tens to hundreds of thousands of spectra or kinetics traces. The data can be acquired in any of several optical modes (absorption, fluorescence, or reflectance) with the additional dimension of time. Data are sorted based on criteria selected by the user and displayed as color-coded lines in a “contour plot,” which makes them easier to view (see Figure 18.3). Sorting criteria include, for example,

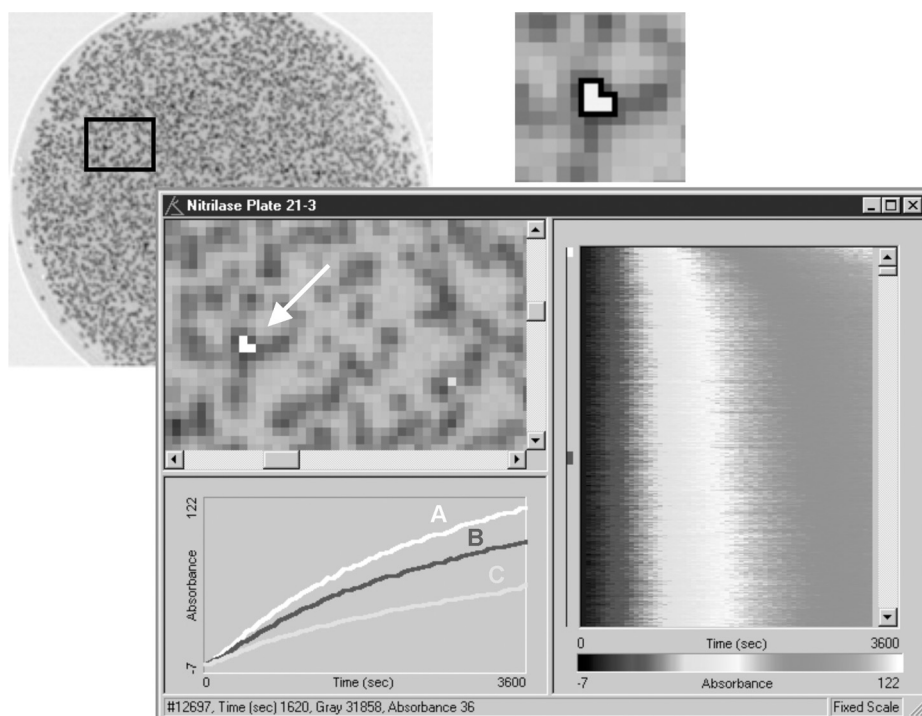


FIGURE 18.3 (See color insert following page 526) Operation of the Kcat graphical user interface (GUI). Data were obtained during screening of a nitrilase library. In the background is an image of the original assay disk, with the region appearing in the GUI enclosed in a rectangle. A single “high-activity” microcolony is also magnified.

wavelength of maximum absorbance, maximum absorbance at a given wavelength, ratio of absorbance at two wavelengths, and maximum absorbance at a given time. Similarly, derivatives or logarithmic plots of the data can also be generated. When the user selects the appropriate elements from the contour plot by clicking and dragging a computer mouse, the corresponding microcolonies are simultaneously highlighted in an image of the assay disk to facilitate retrieval.

Due to the very high density of microcolonies that can be attained on the disks, a throughput of more than 1 million variants per instrument per day is possible. The flexibility of the format makes it feasible to use cells that are intact or lysed, or that secrete the enzyme. The solid-phase format also provides additional operational advantages. For example, because the number of microcolonies per disk can be up to 50,000 or more and the cells that comprise each microcolony occupy a tiny volume, the amount of substrate solution required per microcolony is only about 50 nL. In addition, since the cells are randomly deposited and the substrate is delivered by diffusion, the sample handling is vastly simplified: pipetting is virtually unnecessary and robotics are not required. Likewise, since the microcolonies are placed in contact with the substrate by simply laying the assay disk onto the wick, high-molecular weight substrates, such as viscous polymer suspensions that are incompatible with automated dispensing, can be used. This type of screening cannot be performed using FACS because the polymers do not penetrate the cells.

Additional levels of complexity can be incorporated into solid-phase assays in order to perform more sophisticated screening procedures. For example, the assay disk can be transferred from one environment or chemical pretreatment to another without disturbing the microcolonies. This makes it possible to evolve multiple properties simultaneously by exposing the cells to heat treatments, pH changes, and inhibitors. On the detection side, the ability to obtain complete spectra during the reaction makes it possible to multiplex the substrates, a feature that is useful for changing the enzyme specificity [19,25]. This can be combined with the time-based information in order to identify variants with enhanced activity as well as altered substrate specificity [19,25,29].

18.5 SYNTHESIS OF PHARMACEUTICAL INTERMEDIATES: NITRILASE

The application of enzymes to pharmaceutical synthesis has been an active area of development for many years. Enzymes and whole-cell biocatalysts provide an attractive alternative to conventional chemical synthesis in situations where high regio- or enantioselectivity is required, or where elimination of chemical waste is a major concern [26].

Nitrilases have attracted attention as catalysts for pharmaceutical production because several important drug compounds are carboxylic acids with chiral centers. Examples of these compounds include Lipitor (atorvastatin), a cholesterol-lowering drug, and (*S*)-(+)-ibuprofen, an analgesic. Although carboxylic acids can be produced using conventional chemistry by hydrolyzing their corresponding nitriles, this process requires harsh chemicals. The chemical waste products must also be processed and disposed of. Nitrilase-catalyzed synthesis also offers the possibility of selectively generating either the (*R*)- or (*S*)-enantiomer from the nitrile precursors, and this enantioselectivity could be advantageous for producing a high yield of pure product [26,27].

Naturally occurring nitrilases, however, are not necessarily optimized for pharmaceutical processing conditions. For example, they might be inactive on unusual substrates or in concentrated solutions, and they might not be stable over long periods of time. Introducing large-scale changes to the structure and function of the enzyme is likely to require screening of large libraries of mutants, and in order to perform these directed evolution procedures

efficiently, a high-throughput screening system is also required. We have demonstrated how this could be accomplished in solid phase with some preliminary measurements on a mutagenized bacterial nitrilase gene library.

The basic experiment was performed as follows. Error-prone PCR mutagenesis was used to create a random library of nitrilase variants expressed from a pUC vector in *E. coli*. Microcolonies were grown on assay disks as described in [Section 18.4](#), and a colorimetric assay to detect ammonia (a by-product of nitrilase activity) was performed at 37°C. The increase in the amount of colored product in each microcolony was detected by monitoring the absorbance at 580 nm over time (60 s intervals).

[Figure 18.3](#) shows the Kcat graphical user interface (GUI) after performing a comparative analysis on microcolonies displaying three different levels of nitrilase activity. Approximately 9,000 microcolonies were screened in this experiment on a 47 mm-diameter assay disk using the Kcat instrument and software, and approximately 15,000 kinetics traces were sorted and displayed. The Kcat software was used to sort the kinetics traces pixel by pixel and display them as thin horizontal lines in the contour plot window (right). The horizontal axis in this window is time. In the actual GUI, the intensity of the absorbance at each time point is color-coded from black or blue (low) to pink or white (high), based on the color scale at the bottom. Hence, some of the details are not discernible in this black-and-white figure. See color insert on page xxx.

Pixels showing the greatest change in absorbance over time are sorted to the top. By clicking and dragging the computer mouse over three different regions of the contour plot window, pixels representing high, medium, and low activity were selected. These pixels are identified by color-coded markings (grouping bars) to the left of the contour plot window. (The lowest grouping bar is visible after scrolling.) Several different microcolonies corresponding to these selected pixels are automatically highlighted in the image window (top left). The average kinetics for each of the three classes are also automatically displayed in conventional form in the plot window (lower left). By assigning a measurable activity to particular microcolonies, the variants having the highest apparent activity can then be retrieved from the assay disk for further characterization. [Figure 18.4](#) shows in greater detail how the sorting process operates on kinetics traces to identify the most active microcolonies on the assay disk.

18.6 MODIFICATION OF BIOPOLYMERS: CELLULASE AND OTHER CARBOHYDRATE ENZYMES

Biopolymers encompass an enormous variety of different molecules produced by enzymatic synthesis, including nucleic acids, proteins, polysaccharides, and polyhydroxyalkanoic acids (PHAs) [28]. The versatility of these compounds can be further extended by chemical or enzymatic modification to enhance their functional properties. This makes them highly valuable in industrial, medical, and biotechnological applications. Examples of important applications include paper and construction (cellulose), biopharmaceuticals (antibodies and hormones), biodegradable packaging (PHAs), drug delivery (polylactic acid), tissue engineering (hyaluronan), and ethanol (lignocellulosic biomass).

The challenge for researchers who are screening enzymes for the purpose of modifying large biopolymers is that the substrates may be insoluble in aqueous buffer or they may form highly viscous suspensions. These properties can make them difficult to handle in conventional liquid-phase systems. For example, in screening for variants of galactose oxidase with enhanced activity on guar (a galactomannan polymer), it was desirable in some assays to use a suspension of 2% guar [29]. This material is very difficult to pipette due to its high viscosity. However, using the solid-phase format, it was possible to mix the suspension with 2% agarose

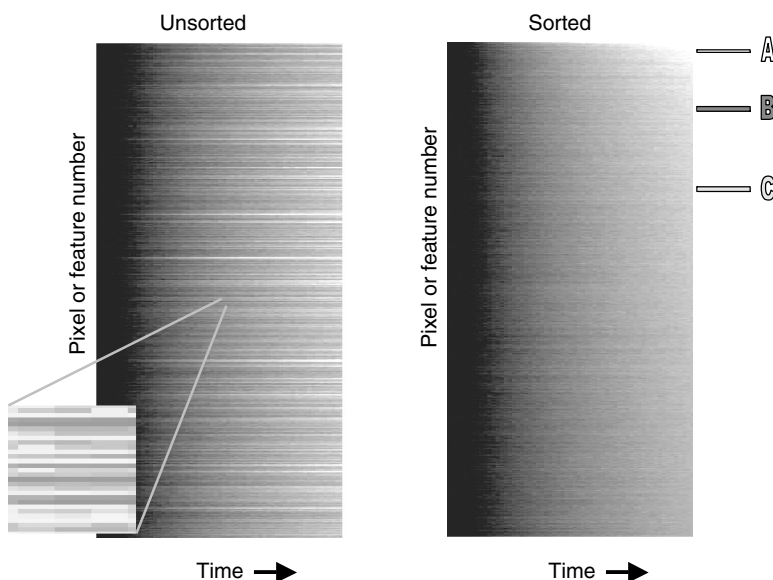


FIGURE 18.4 (See color insert following page 526) Sorting nitrilase kinetics using the contour plot window. All 15,000 kinetics traces are visible in each view. The data are the same as that of [Figure 18.3](#). The x -axis corresponds to elapsed time, and the y -axis to pixel or feature number. In the actual window, the intensity of the absorption at a given time is color-coded. The unsorted traces on the left display a random color pattern. The traces on the right have been sorted based on their change in absorbance over time. The most “active” pixels are thereby moved to the top. Using the computer mouse, it is possible to select various groups of pixels (marked by the three grouping bars to the right of the window) to identify the corresponding microcolonies.

and then overlay an assay disk containing the microcolonies. This made it feasible to screen for activity directly on the target substrate. A variant with 16-fold higher activity was ultimately identified. Variants with improved thermostability were also isolated using solid-phase screening [19].

Another key enzyme class involved in modifying biopolymers is cellulase, which includes cellobiohydrolase and endoglucanase. Cellobiohydrolase hydrolyzes crystalline cellulose by sequentially removing cellobiose units from the ends of the cellulose chains. Endoglucanase randomly cleaves cellulose within the amorphous regions of the polymer to create chains of various lengths [30]. These enzymes have important applications in pulp and paper processing, textile finishing, and converting agricultural waste into fermentable sugars. Enhanced thermostability is one of the desired goals for cellulase engineering.

Quantitative, high-throughput assays for pulp and paper cellulases are difficult to design because of the high molecular weight and insolubility of the cellulose. In some cases, the actual substrate is wood pulp, which contains other polymeric constituents, such as hemicelluloses and lignins. For these reasons, many laboratories screening for endoglucanase activity employ surrogate substrates, such as *p*-nitrophenyl cellotrioside (PNP-cellotrioside, which releases a yellow chromophore upon hydrolysis), or azurine cross-linked hydroxyethyl-cellulose (AZCL-HE cellulose, an insoluble fibrous material that releases a blue chromophore upon hydrolysis). In the case of PNP-cellotrioside, the reaction turns from colorless to yellow. However, in the case of AZCL-HE cellulose, the starting material is already colored, and the progress of the reaction is monitored by measuring release of the dye from the cellulose matrix. The latter substrate has the advantage of being more similar to the natural

cellulose fiber than is the trisaccharide, but it is inconvenient to use in liquid phase because the dye released into solution must be separated from the dye that is still attached to the cellulose fibers. An analogous separation procedure must also be performed on the soluble version of the dyed substrate.

In solid phase, the task of identifying the active microcolonies is somewhat easier. In the presence of AZCL-HE cellulose (dispersed in an agarose matrix), activity is indicated by the development of clearing zones in the agarose underneath the microcolonies. By imaging the assay disk at 590 nm over time, it is possible to monitor the growth of these clearing zones to find variants with the highest activity. Variants with improved thermostability can be screened by subjecting the assay disk to heating for a fixed time prior to performing the assay. An example of screening on AZCL-HE cellulose is shown in Figure 18.5. Preliminary screening of an endoglucanase library yielded a variant that showed a 15°C improvement in thermostability compared to the parental enzyme after pretreatment at various temperatures for 15 min. Ongoing software improvements will make it possible to quantitatively analyze

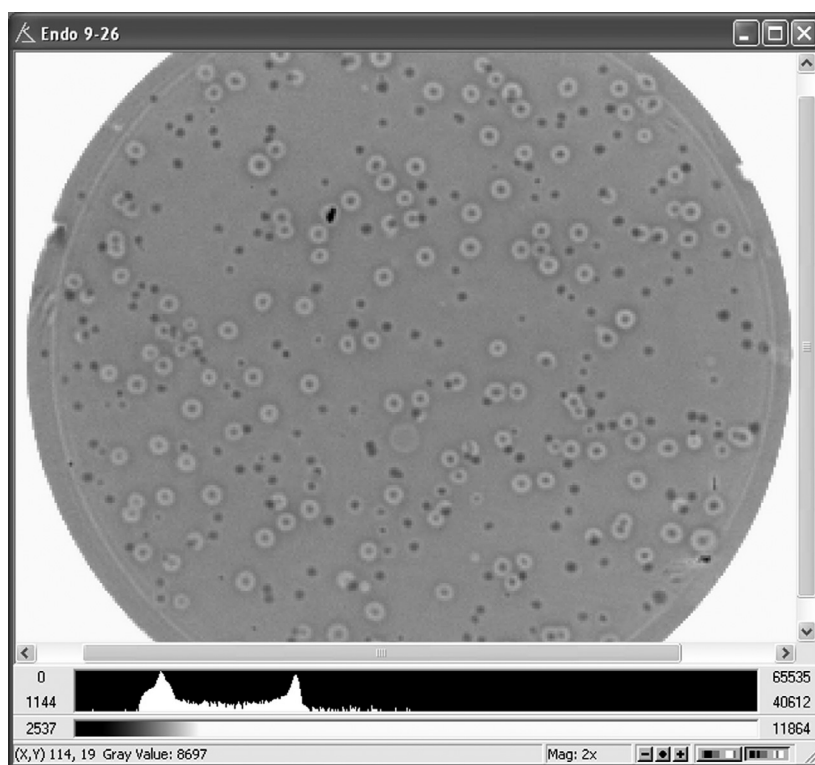


FIGURE 18.5 Thermostability screening of a library of endoglucanase mutants assayed on azurine cross-linked hydroxyethylcellulose (AZCL-HE cellulose) dispersed in agarose. Microcolonies on the assay disk were exposed to 57°C heat treatment for 15 min. The assay for thermal stability was then performed on the substrate at 37°C to detect those mutants with the highest residual activity. The image was obtained at 550 nm in the Kcat instrument. Microcolonies containing active endoglucanase variants hydrolyze the labeled substrate and release the bound dye. Combined diffusion of enzyme and dye creates the characteristic “donut” shape around the active microcolonies in this image. The degree of intensity loss at this time point is approximately proportional to the enzyme activity remaining after the heat treatment. The kinetics of clearing zone formation can be used to identify microcolonies expressing the most active enzyme variants.

the clearing zones and increase the microcolony density. This will benefit all types of assays on carbohydrate polymers that employ the dye release method, including assays for xylanase, mannanase, pullulanase, and amylase.

18.7 FUTURE PROSPECTS

18.7.1 ADVANTAGES OF CUSTOMIZED ENZYMES

The ability to perform very high-throughput screening with a user-friendly, bench-top device will make it possible for individual laboratories to create their own custom enzymes for specific applications. From the industrial process perspective, this means that companies can develop biocatalysts that are highly compatible with their own proprietary chemistry, rather than having to rely on commercial enzyme products that were designed for a different purpose. This new capability will hopefully broaden the range of applications for industrial enzymes.

18.7.2 GREEN CHEMISTRY FOR CHEAPER, CLEANER PHARMACEUTICAL MANUFACTURING

In the area of pharmaceutical and specialty chemical manufacturing, introducing custom enzyme catalysts will provide more options to reduce hazardous waste, streamline complex synthetic processes, and create new chemical entities. Toolboxes of enzymes will be available to provide alternative routes to conventional organic synthesis. Production cost savings and added functional value will enable the users to remain profitable despite increasing competition.

18.7.3 VALUE OF MODIFIED POLYMERS

In the area of polymer chemistry, the availability of customized enzymes will enable chemists to generate novel functionalized polymers. These new compounds will provide exotic materials that are both biocompatible and biodegradable. Moreover, their performance characteristics can be fine-tuned to specific applications. More efficient production of these products will also save energy, reduce pollution and greenhouse gas emissions, and decrease our reliance on petrochemical feedstocks.

REFERENCES

1. Hugenholtz, P., Goebel, B.M., and Pace, N.R., Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity, *J. Bacteriol.*, 180, 4765, 1998.
2. Streit, W.R., Daniel, R., and Jaeger, K.E., Prospecting for biocatalysts and drugs in the genomes of non-cultured microorganisms, *Curr. Opin. Biotechnol.*, 15, 285, 2004.
3. Brennan, Y. et al., Unusual microbial xylanases from insect guts, *Appl. Environ. Microbiol.*, 70, 3609, 2004.
4. Bommarius, A.S. and Riebel, B.R., Eds., *Biocatalysis*, Wiley-VCH, Weinheim, Germany, 2004.
5. Holland, J.H., Genetic algorithms, *Sci. Amer.*, 267, 66, 1992.
6. Arkin, A.P. and Youvan, D.C., An algorithm for protein engineering: simulations of recursive ensemble mutagenesis, *Proc. Natl. Acad. Sci. USA*, 89, 7811, 1992.
7. Arkin, A.P. and Youvan, D.C., Optimizing nucleotide mixtures to encode specific subsets of amino acids for semi-random mutagenesis, *Biotechnology* (New York), 10, 297, 1992.
8. Youvan, D.C., Arkin, A.P., and Yang, M.M., Recursive ensemble mutagenesis: a combinatorial optimization technique for protein engineering, in *Parallel Problem Solving from Nature II. Proceedings of the Second International Conference on Parallel Problem Solving from Nature*, Maenner, R. and Manderick, B., Eds., Elsevier Publishing, Amsterdam, 1992, p. 401.
9. Delagrave, S., Goldman, E.R., and Youvan, D.C., Recursive ensemble mutagenesis, *Protein Eng.*, 6, 327, 1993.

10. Delagrave, S. and Youvan, D.C., Searching sequence space to engineer proteins: exponential ensemble mutagenesis, *Biotechnology* (New York), 11, 1548, 1993.
11. Stemmer, W.P.C., DNA shuffling by random fragmentation and reassembly: *in vitro* recombination for molecular evolution, *Proc. Natl. Acad. Sci. USA*, 91, 10747, 1994.
12. Stemmer, W.P.C., Rapid evolution of a protein *in vitro* by DNA shuffling, *Nature*, 370, 389, 1994.
13. Chen, K.Q. and Arnold, F.H., Enzyme engineering for nonaqueous solvents: random mutagenesis to enhance activity of subtilisin E in polar organic media, *Biotechnology* (New York), 9, 1073, 1991.
14. Tobin, M.B., Gustafsson, C., and Huisman, G.W., Directed evolution: the 'rational' basis for 'irrational' design, *Curr. Opin. Struct. Biol.*, 10, 421, 2000.
15. Peng, W. et al., Analytical study of the effect of recombination on evolution via DNA shuffling, *Phys. Rev. E Stat. Nonlin. Soft Matter Phys.*, 69, 051911, 2004.
16. Moore, G. et al., Predicting crossover generation in DNA shuffling, *Proc. Natl. Acad. Sci. USA*, 98, 3226, 2001.
17. Joern, J.M., Meinhold, P., and Arnold, F.H., Analysis of shuffled gene libraries, *J. Mol. Biol.*, 316, 643, 2002.
18. Maheshri, N. and Schaffer, D.V., Computational and experimental analysis of DNA shuffling, *Proc. Natl. Acad. Sci. USA*, 100, 3071, 2003.
19. Delagrave, S. et al., Combinatorial mutagenesis algorithms, digital imaging spectroscopy, and solid-phase assays for directed evolution, in *Enzyme Functionality: Design, Engineering and Screening*, Svendsen, A., Ed., Marcel Dekker, New York, 2004, p. 507.
20. Morley, K.L. and Kazlauskas, R.J., Improving enzyme properties: when are closer mutations better? *Trends Biotechnol.*, 23, 231, 2005.
21. Cohen, N. et al., *In vitro* enzyme evolution: the screening challenge of isolating the one in a million, *Trends Biotechnol.*, 19, 507, 2001.
22. Wittrup, K.D. and Bailey, J.E., A single-cell assay of beta-galactosidase activity in *Saccharomyces cerevisiae*, *Cytometry*, 9, 394, 1988.
23. Olsen, M.J. et al., Function-based isolation of novel enzymes from a large library, *Nat. Biotechnol.*, 18, 1071, 2000.
24. Griffiths, A.D. and Tawfik, D.S., Directed evolution of an extremely fast phosphotriesterase by *in vitro* compartmentalization, *EMBO J.*, 22, 24, 2003.
25. Bylina, E.J. et al., Solid-phase enzyme screening, *ASM News*, 66, 211, 2000.
26. Sheldon, R.A. and van Rantwijk, F., Biocatalysis for sustainable organic synthesis, *Austral. J. Chem.*, 57, 281, 2004.
27. Robertson, D.E. et al., Exploring nitrilase sequence space for enantioselective catalysis, *Appl. Environ. Microbiol.*, 70, 2429, 2004.
28. Gross, R.A. and Cheng, H.N., Eds., *Biocatalysis in Polymer Science*, vol. 840, ACS Symposium Series, American Chemical Society, Washington, DC, 2003.
29. Delagrave, S. et al., Application of a very high-throughput digital imaging screen to evolve the enzyme galactose oxidase, *Protein Eng.*, 14, 261, 2001.
30. Hilden, L. and Johansson, G., Recent developments on cellulases and carbohydrate-binding modules with cellulose affinity, *Biotechnol. Lett.*, 26, 1683, 2004.

19 Deaminating Enzymes of the Purine Cycle as Biocatalysts for Chemoenzymatic Synthesis and Transformation of Antiviral Agents Structurally Related to Purine Nucleosides

Enzo Santaniello, Pierangela Ciuffreda, and Laura Alessandrini

CONTENTS

19.1	Introduction.....	502
19.2	Deaminating Enzymes of the Purine Cycle	503
19.3	Structure and Catalytic Mechanism of Purine Nucleoside and Nucleotide Deaminases	503
19.4	Adenosine Deaminase as Biocatalyst.....	504
19.5	Adenylate Deaminase as Biocatalyst	505
19.6	ADA-Assisted Chemoenzymatic Synthesis and Transformation of Antiviral Nucleosides	507
	19.6.1 2',3'-Dideoxygenated Purine Nucleosides	507
19.7	Fluorine-Containing Nucleosides	509
19.8	Nucleosides Containing Substitutions in the Furanose Moiety.....	511
19.9	Oxetanocin A.....	511
19.10	ADA-Assisted Chemoenzymatic Synthesis of Antiviral Carbocyclonucleosides....	512
19.11	ADA-Assisted Chemoenzymatic Synthesis of Antiviral Acyclonucleosides.....	514
19.12	AMPDA-Assisted Synthesis and Transformation of Antiviral Nucleosides and Analogs	515
19.13	Stereoselectivity of Deaminating Enzymes.....	516
19.14	Resolution of Synthetic Nucleosides and Analogs.....	517
19.15	Diastereoselectivity of Deaminating Enzymes.....	521
19.16	Concluding Remarks.....	522
	References	523

19.1 INTRODUCTION

Many synthetic compounds structurally related to natural nucleosides are characterized by biopharmacological effects that include anticancer [1,2] or antiviral [3,4] activities. Several compounds are effective against viruses that are causative agents of severe infections, such as the human immunodeficiency virus (HIV) [5,6]. Synthetic antiviral compounds that are structurally related to nucleosides may be characterized by modifications in the carbohydrate or base moiety, while substitution of the furanose ring with an analogous cyclopentane system generates carbocyclic nucleosides. In these compounds, the problem associated with the presence of the β -*N*-glycosidic bond, making nucleoside structure vulnerable to chemical or enzymatic hydrolysis and favoring loss of pharmacological activity in a nucleosidic drug, is eliminated. Only a few carbocyclonucleosides are naturally occurring, such as aristeromycin and neplanocin that have been isolated from microbial fermentation [7,8]. In general, carbocyclonucleosides are prepared by chemical total synthesis that sometimes uses chemoenzymatic methodologies [9–11]. Many synthetic acyclic analogs of nucleosides (acyclonucleosides) also present important antiviral activity and for the synthesis of two leading products such as acyclovir and ganciclovir chemical and chemoenzymatic procedures have already been reviewed [12].

Due to the importance of modified nucleosides and analogs in medicinal chemistry, the development of synthetic methodologies for the selective modification of natural nucleosides or for the preparation of related compounds has been extensively investigated and many efficient procedures are currently available. A special place is occupied by the methods that take advantage of the great potential that enzymes possess as biocatalysts for reactions of synthetic relevance and in this well-established field of research a few reviews deal with enzyme-catalyzed modifications of nucleosides and related compounds [13–15]. The specific topic of the enzymatic synthesis of antiviral agents was reviewed a few years ago [16] and this work includes also nucleosides and structurally related compounds. Adenosine deaminase (adenosine aminohydrolase, ADA, EC 3.5.4.4) and adenylate deaminase (5'-adenylic acid deaminase, AMP deaminase, AMPDA, EC 3.5.4.6) are enzymes of the purine cycle that catalyze the hydrolytic deamination of purine nucleosides and nucleotides [17]. Recently, it has been shown that these deaminating enzymes are able to transform a great number of modified nucleosides or related analogs and their potential as biocatalysts in nucleoside chemistry has been recently reviewed [18].

This chapter will cover applications of the above deaminases to the preparation and transformation of compounds structurally related to nucleosides that are characterized by antiviral activity. It should be pointed out that in the majority of articles dealing with modified nucleosides, positions of the purine ring are indicated by a conventional, rather than systematic numbering that has to be used for different heterocyclic systems related to purine. For the sake of clarity, in Figure 19.1 we have reported both the above criteria for adenosine.

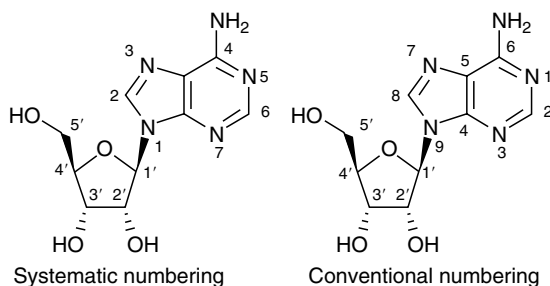


FIGURE 19.1 Systematic and conventional numbering of adenosine.

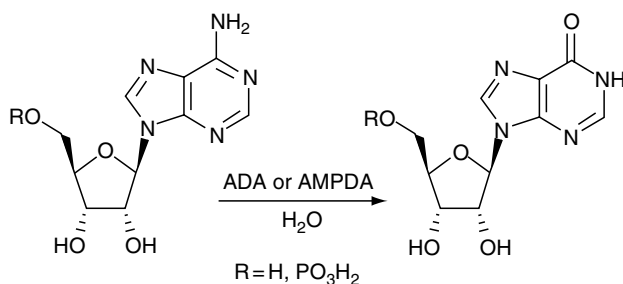


FIGURE 19.2 ADA- and AMPDA-catalyzed hydrolytic deamination of adenosine and adenylic acid.

19.2 DEAMINATING ENZYMES OF THE PURINE CYCLE

ADA and AMPDA belong to the class of hydrolases and are metalloenzymes whose activity depends on the presence of a Zn^{2+} cation [17]. ADA catalyzes the deamination of adenosine to inosine and AMPDA converts 5'-phosphate adenosine (adenylic acid, AMP) to inosine 5'-phosphate (Figure 19.2).

ADA is present in microorganism, invertebrate, and mammalian cells, including human, where its role at physiological and pathological level has been thoroughly investigated, also with the aim of establishing a gene therapy for specific diseases and designing of enzyme inhibitors [19]. The role of AMPDA has been mainly investigated at muscular level and it is now well established that it is one of the principal enzymes of the purine nucleotide cycle [20] that concurs to the regulation of the level of adenosine.

Both enzymes are commercially available at a considerable level of purity and adequate activity, thus ADA and AMPDA are potential biocatalysts for chemoenzymatic transformations in nucleoside chemistry. Calf intestinal mucosa is the main source of commercial ADA (Sigma, type II, 1 to 5 U/mg protein) and AMPDA from *Aspergillus* sp. is the enzymatic preparation used in food industry for large-scale production of inosine 5'-phosphate (inosinic acid), an important meat-like flavor [21]. The fact that these enzymes are hydrolases constitutes an additional advantage in their use as biocatalysts, because they do not require additional coenzymes for their catalytic action. Consequently, these deaminating enzymes do not present the inconvenience related to recycling of the cofactor, a major limitation in the use of coenzyme-dependent biocatalysts [22].

19.3 STRUCTURE AND CATALYTIC MECHANISM OF PURINE NUCLEOSIDE AND NUCLEOTIDE DEAMINASES

No studies on three-dimensional structure of AMPDA have been reported yet, whereas, since the first report on crystallized murine enzyme, a well-established set of structural information is currently available for ADA [23]. All x-ray studies have been carried out on ADA and inhibitor complexes [23–26] and, from the available structural data, a well-defined enzyme-catalyzed mechanism can be drawn for hydration of adenosine, as shown in Figure 19.3. Water attack on position 6 of the purine ring is assisted by His-238, Asp-295, and Zn^{2+} to form a tetrahedral intermediate that irreversibly eliminates ammonia with the formation of inosine.

Although structural data for AMPDA are not available, it is generally accepted that its deaminating activity should follow the same mechanism shown in Figure 19.3 for ADA, as evidenced by the fact that nucleoside-like inhibitors of ADA, after *in vivo* phosphorylation of the 5'-hydroxy group, tend to inhibit AMPDA as well [27].

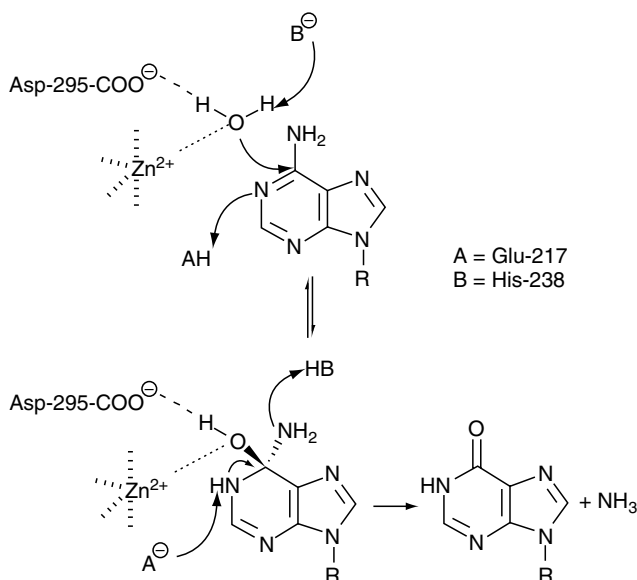


FIGURE 19.3 Mechanism of ADA-catalyzed hydrolytic deamination.

19.4 ADENOSINE DEAMINASE AS BIOCATALYST

Purified ADA from calf intestinal mucosa has been used for the biotransformation of a wide range of structurally modified purine nucleosides. The enzyme is relatively stable also in the presence of polar cosolvents [28], which may overcome problems related to the solubility of modified nucleosides into the reaction medium constituted by water or buffer solutions. Works on kinetic determination of ADA activity with different substrates have outlined a clear picture of the enzyme specificity. In addition to the physiological hydrolytic deamination of adenosine, ADA is able to transform into inosine a few other 6-substituted purine ribosides shown in Figure 19.4, although at a slower rate [29–31].

Also ADA from bovine placenta confirmed the above-described specificity with respect to the hydrolysis of various 6-substituted adenosines [32]. Introduction of additional groups at position 2, such as alkylamino, alkylthio, and halogen [32] as well as nitro [33] caused a competitive inhibition of the enzyme. The activity of ADA on adenosines modified in the furanose moiety has been studied on kinetic basis [34] and interesting conclusions on the relationship between substrate structure and enzyme activity were drawn [35]. Studying

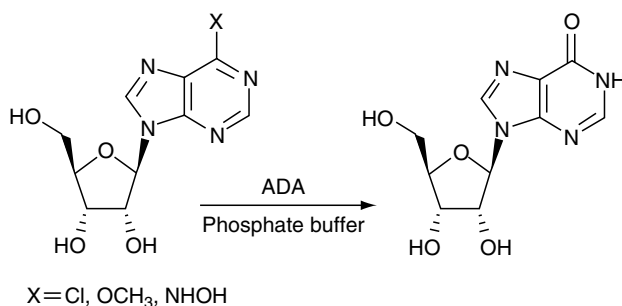


FIGURE 19.4 ADA-catalyzed transformation of 6-substituted purine nucleosides.

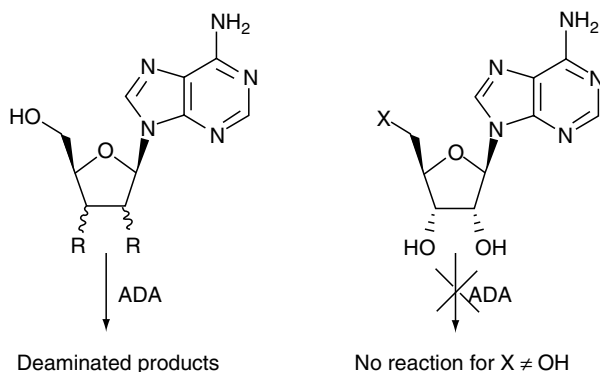


FIGURE 19.5 ADA activity on adenosines bearing modifications in the furanose moiety.

several structurally different substrates, including ribose epimers, it was demonstrated that protection, substitution, or elimination of 2'- and 3'-hydroxy groups were compatible with the enzyme activity. 5'-Protected or 5'-deoxy-5'-substituted nucleosides were not substrates (Figure 19.5) and the overall results suggested that the presence of the 5'-hydroxy group in any modified adenosine or 6-substituted purine nucleoside is essential for ADA activity.

Further, extensive investigation on another series of adenine nucleoside derivatives confirmed the crucial role of the 5'-hydroxymethyl function [36]. Apparent exception to the above statement could be represented by the deamination of 5'-deoxyadenosine [37], but long reaction time was required and yields were not clearly indicated. In the claimed conversion of adenosine 5'-deoxy-5'-thioether to the corresponding inosine derivative with ADA from *Aspergillus oryzae* [38], probably the enzyme was not adenosine but adenylyate deaminase, as it has recently been suggested [39]. The fundamental importance of the 5'-hydroxy group was evidenced by the results obtained from the ADA-catalyzed deamination of acetates of adenosine and 2'- or 3'-deoxyadenosine [40].

In a reinvestigation of ADA-catalyzed deamination of 5'-protected or 5'-deoxy-5'-substituted 2',3'-isopropylidene adenosines (Figure 19.6), the crucial role of the 5'-hydroxy group was confirmed with the only exception of slow reacting 5'-deoxy-5'-amino derivative [41]. It was also shown that the previously reported use of DMSO as cosolvent [42] did not significantly influence the deamination rate.

19.5 ADENYLATE DEAMINASE AS BIOCATALYST

As mentioned in the introduction, an AMPDA from the microorganism *Aspergillus* sp. is commercially available in bulk quantity for its use in large-scale production of inosine 5'-phosphate for food industry [21]. In spite of this availability, the enzyme has been applied only recently to the synthesis of 6-oxopurine nucleosides and their analogs [43]. In this report, Margolin and coworkers determined the relative rates of deamination of various modified nucleosides, including 6-substituted ribonucleosides, in the presence of AMPDA, comparing their data with those obtained using ADA as reference deaminating enzyme. Interestingly, AMPDA was able to deaminate also 3',5'-cyclic monophosphate adenosine and compounds such as (4'*E*)- and (4'*Z*)-4',5'-didehydro-5'-deoxy-5'-fluoroadenosine, 4',5'-didehydro-5'-deoxyadenosine, and 5'-deoxy-2',3'-*O*-(1-methylethylidene)-5'-(phenylsulfonyl) adenosine. These compounds were not substrates for ADA, thus indicating that for AMPDA the structural restriction related to the presence of the 5'-OH in the substrate was not as mandatory as for ADA (Figure 19.7).

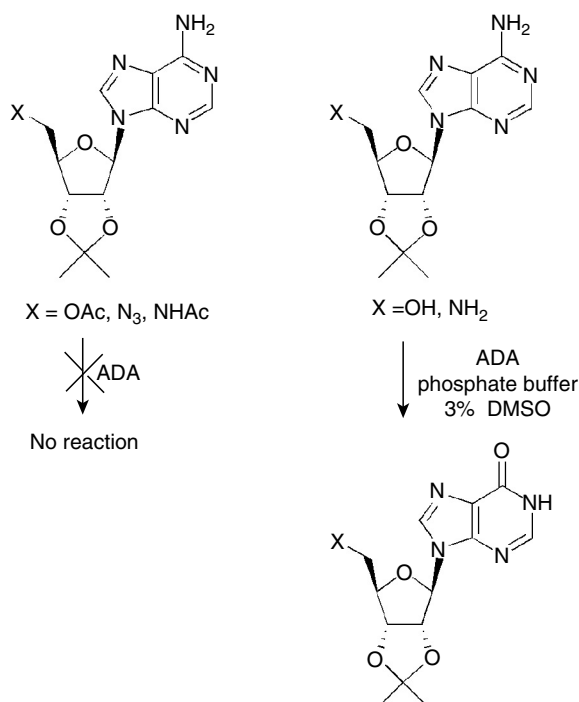


FIGURE 19.6 ADA-catalyzed deamination of 5'-protected or 5'-deoxy-5'-substituted 2',3'-isopropylidene of the adenosines.

This was later confirmed by the observation that a few 5'-protected or 5'-deoxy-5'-substituted adenosines (Figure 19.8), whose ADA was not able to transform or that were only poorly deaminated, were completely converted into inosine derivatives with AMPDA at room temperature in 3 to 150 min [39].

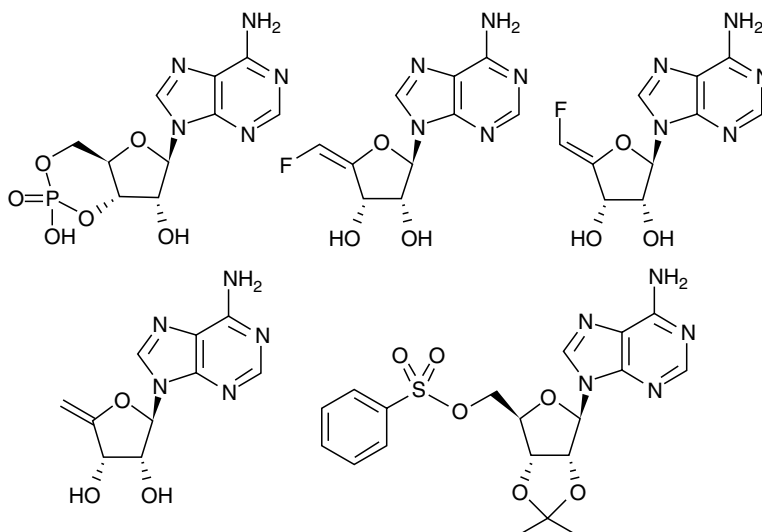


FIGURE 19.7 Substrates for AMPDA-catalyzed deamination.

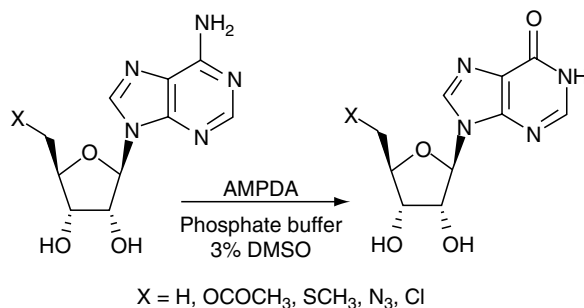


FIGURE 19.8 AMPDA-catalyzed deamination of 5'-protected and 5'-deoxy-5'-substituted adenosines.

19.6 ADA-ASSISTED CHEMOENZYMATIC SYNTHESIS AND TRANSFORMATION OF ANTIVIRAL NUCLEOSIDES

The above reported data on the activity of the enzymes constitute an important background for the rational use of ADA and AMPDA as biocatalyst in the preparation of modified nucleosides. Here, we will review the application of ADA to the synthesis and elaboration of nucleosides containing modified purine systems, adenosines with modification of the ribose (or 2'-deoxyribose) moiety, and synthetic nucleosides containing structural variations on the part of both purine and furanose. Substrates of the deaminating enzyme will be selected within the general framework of a recognized or potential antiviral activity.

19.6.1 2',3'-DIDEOXYGENATED PURINE NUCLEOSIDES

Purine nucleosides that are deoxygenated at 2' and 3' positions are known inhibitors of HIV reverse transcriptase, a key enzyme for the retrovirus replication [44] and a few of them have been introduced as chemotherapeutic agents with antiretroviral activity [5]. For instance, 2',3'-dideoxyinosine (ddI) is the drug Didanosine used for the treatment of HIV advanced infection [45], and this modified nucleoside can be prepared by a few synthetic approaches [46,47]. A chemoenzymatic route to the synthesis of ddI that relies on ADA-catalyzed deamination of 2',3'-dideoxyadenosine (ddA) has been reported [48] and shown in Figure 19.9.

Another chemoenzymatic synthesis of ddI has been recently proposed [49] and consisted in ADA-catalyzed deamination of 2'-deoxyadenosine to 2'-deoxyinosine and in a selective 5'-acetylation of this compound catalyzed by the lipase from *Candida cylindracea* (CAL). Deoxygenation of the unprotected 3'-hydroxy group was chemically achieved by a classical approach [50] that consisted in the preparation of the 3'-*O*-phenoxythiocarbonyl derivative and in further reaction with tributyltin hydride (Figure 19.10).

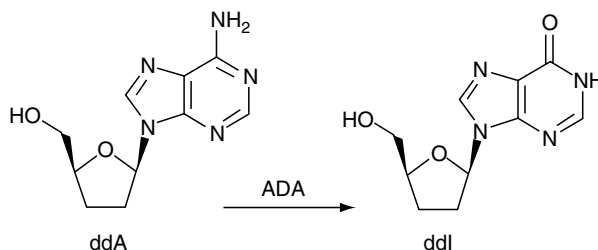


FIGURE 19.9 ADA-catalyzed deamination of 2',3'-dideoxyadenosine to 2',3'-dideoxyinosine.

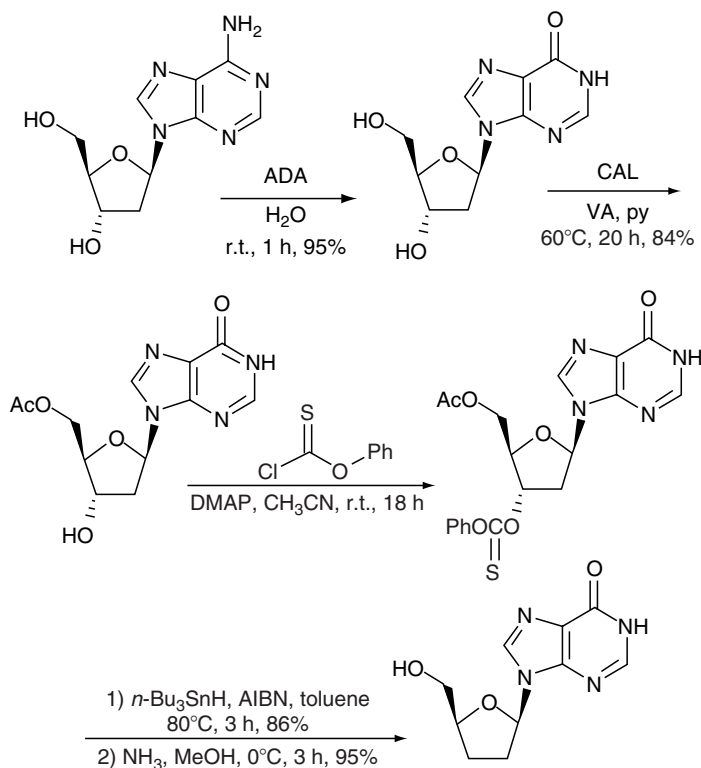


FIGURE 19.10 Chemoenzymatic synthesis of 2',3'-dideoxyinosine from 2'-deoxyadenosine.

A series of 8-substituted 2',3'-dideoxyadenosines, synthesized as potential inhibitors of HIV reverse transcriptase, were characterized by higher stability than 8-unsubstituted analogs [51]. The deaminating activity of ADA that had previously been evaluated on other 8-substituted adenosines [52] was applied to 8-amino and 8-hydroxy derivatives that were converted into the corresponding inosines (4 and 12 h, respectively). 8-Methylthio, 8-methoxy, and 8-benzyloxy derivatives were resistant to the enzymatic deamination (Figure 19.11).

In addition, 8-aza-7-deaza-2',3'-dideoxyadenosine, synthesized as a potential antiretroviral agent, was deaminated by ADA [53], as shown in Figure 19.12.

Although 2-substituted adenosines have been described as inhibitors of ADA [31], the presence of an amino group at position 2 is compatible with the activity of the enzyme, as shown by the slow deamination of 2-aminoadenosine to guanosine [54]. Therefore, a 2,6-diaminopurine can be considered a masked guanosine system and its ADA-catalyzed deamination may offer an alternative route to direct chemical transformation of guanosine systems that is plagued by experimental problems, such as poor solubility, instability, and formation of gel [55,56]. Following the above biocatalytic approach, Robins and coworkers [42] have described the preparation of a few ribose-modified guanine nucleosides and it is worth mentioning that for a substrate with amino groups in positions 2, 6, and 2' as, for instance, 2,6-diamino-9-(2'-amino-2'-deoxy-β-D-arabinofuranosyl)purine, only the selective deamination of the 6-amino group occurs in the presence of ADA (Figure 19.13). This chemoenzymatic approach has been applied to the preparation of 2',3'-dideoxyguanosine, an effective inhibitor of hepatitis B virus [57,58] that can be enzymatically prepared from the corresponding 2,6-diaminopurine riboside, which is the inhibitor of the enzyme HIV and hepatitis B viruses [59–61]. 2',3'-Dideoxyguanosine can be also prepared by ADA-catalyzed deamination

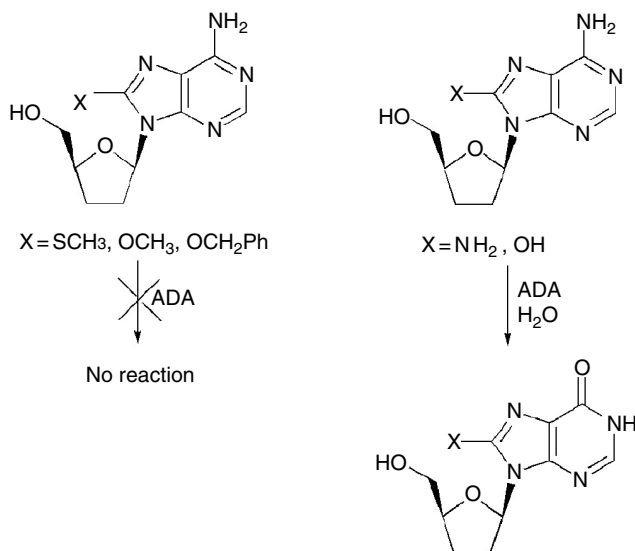


FIGURE 19.11 ADA-catalyzed deamination of 8-substituted 2',3'-dideoxyadenosines.

of 2-amino-6-chloro-2',3'-dideoxyfuranoside [62]. Within a work aimed to select cytotoxic or antihepatitis B modified nucleosides [63], other 2-amino-6-substituted 2',3'-dideoxy nucleosides were deaminated by ADA to the corresponding guanosines with a decreasing efficiency of hydrolysis going from 6-amino to 6-methoxy and to 6-ethoxy nucleosides (Figure 19.13).

19.7 FLUORINE-CONTAINING NUCLEOSIDES

Introduction of a fluorine atom in the carbohydrate ring of nucleosides has been a means to improve the stability of the compound toward enzymatic or chemical hydrolysis of the β -*N*-glycosidic bond. The resistance to acidic pH of 2'-fluoro purine dideoxygenated nucleosides has been, in fact, verified [64]. The 2',3'-dideoxy-2'-ara-fluoroadenosine, conveniently synthesized from 6-chloropurine, was converted to the corresponding inosine derivative by ADA-catalyzed deamination (room temperature, overnight, 77%). In this case, yield of enzymatic reaction was lower than the treatment with sodium nitrite in acetic acid (room temperature, 20 h, 95%; Figure 19.14).

A series of 6-substituted 2'- β -fluoro-2',3'-dideoxypurine nucleosides (Figure 19.15) have been prepared with the aim of finding lipophilic, ADA-activated anti-HIV-prodrugs for the specific delivery in central nervous system [65,66]. The activity of ADA with these substrates has been evaluated and it turned out that the 6-fluoro nucleoside was hydrolyzed to the corresponding inosine with the highest relative rate.

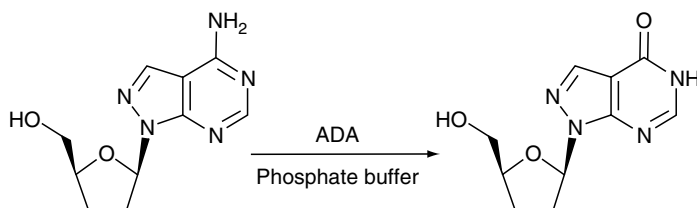


FIGURE 19.12 ADA-catalyzed deamination of 8-aza-7-deaza-2',3'-dideoxyadenosine.

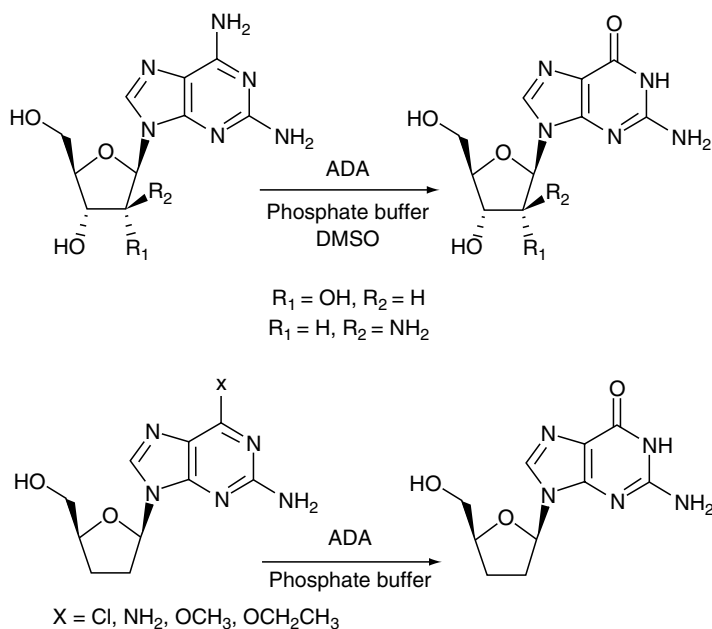


FIGURE 19.13 Selective ADA-catalyzed deamination of 2,6-substituted diaminopurine nucleosides.

A fluoro-*arabino* analog of 8-aza-7-deaza-2',3'-dideoxyadenosine, i.e., 4-amino-1-(2'-deoxy-2'-fluoro- β -D-arabinofurano)-1*H*-pyrazolo[3,4-*d*]pyrimidine, showed activity against a human herpes virus [67]. The enzymatic deamination with ADA is similar to that reported in Figure 19.12 and readily proceeds to afford the corresponding 4-oxo compound (Figure 19.16).

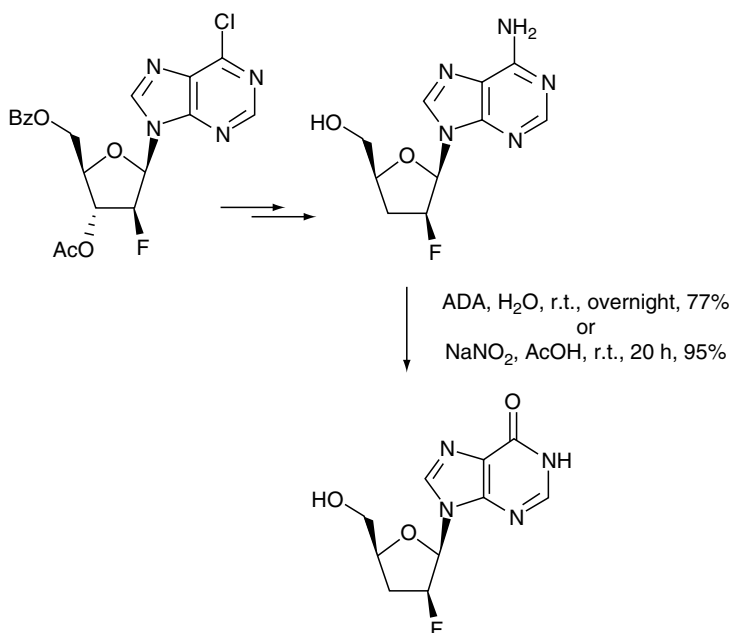


FIGURE 19.14 ADA-catalyzed deamination of 2',3'-dideoxy-2'-ara-fluoroadenosine.

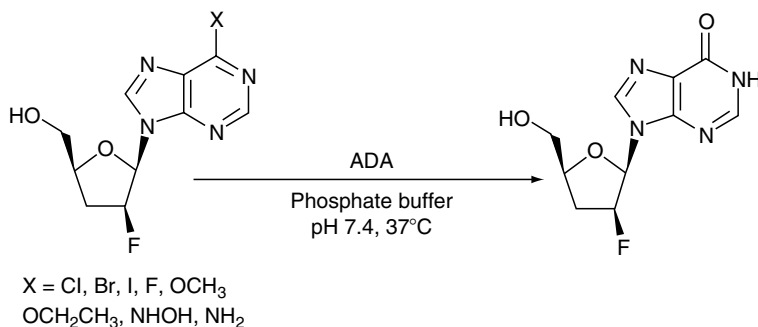


FIGURE 19.15 ADA-catalyzed deamination of 6-substituted 2'- β -fluoro-2',3'-dideoxypurine nucleosides.

19.8 NUCLEOSIDES CONTAINING SUBSTITUTIONS IN THE FURANOSE MOIETY

Numerous nucleosides and 2'-deoxynucleosides substituted at various positions of the furanose moiety show marked activities as antiviral agents, for instance, C-3'-branched deoxynucleosides. For C-3'-ethynyl nucleosides, it has been demonstrated that transformation into the corresponding nucleotides is required to become active on HIV reverse transcriptase [68]. 3'- β -Ethynyladenosine and the corresponding 2'-deoxynucleoside were deaminated by ADA, differently from 3'-vinyl and ethyl substituted compounds that were not substrates (Figure 19.17) [69].

4'-C-Substituted 2'-deoxynucleosides constitute a group of antiviral agents effective against HIV variants that are resistant to other antiretroviral drugs [70]. Previous studies on structure–activity of 4'-C-substituted nucleosides had directed the investigation toward compounds bearing small substituents at 4'-position as the most active compounds [71–73]. The synthesis of 4'-cyano- and 4'-ethynyl-2'-deoxypurine nucleosides was reported [74] and these compounds were shown to be substrate of ADA for the preparation of the corresponding 4'-substituted-2'-deoxyinosine and guanosine, respectively (Figure 19.18).

19.9 OXETANOCIN A

Oxetanocin A is a naturally occurring nucleoside characterized by a peculiar 4-member ring carbohydrate moiety that has been isolated from *Bacillus megaterium* [75] and was soon recognized as a HIV inhibitor [76]. Strictly related to oxetanocin A, the cyclobutyl guanine nucleoside analog, Lobucavir, has been synthesized as an antiviral agent for the treatment

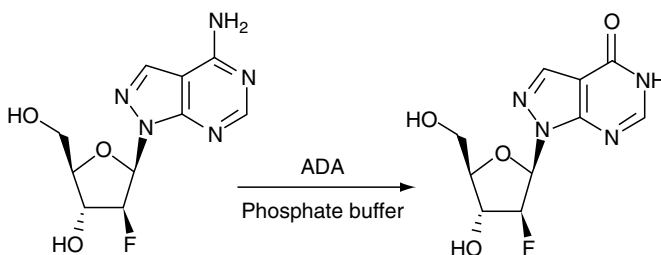


FIGURE 19.16 ADA-catalyzed deamination of 4-amino-1-(2'-deoxy-2'-fluoro- β -D-arabino furanosyl)-1H-pyrazolo [3,4-d]pyrimidine.

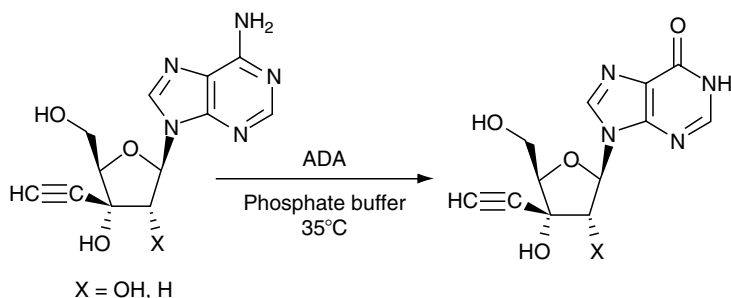


FIGURE 19.17 ADA-catalyzed deamination of 3'-β-ethynyladenosines.

of herpes viruses and hepatitis B [77]. Oxetanocin A and the 2-amino analog are readily and quantitatively converted (Figure 19.19) to the corresponding hypoxanthine and guanosine derivatives by an ADA-catalyzed reaction [78].

19.10 ADA-ASSISTED CHEMOENZYMATIC SYNTHESIS OF ANTIVIRAL CARBOCYCLONUCLEOSIDES

Replacement of the sugar moiety of nucleosides with a cyclopentyl ring leads to carbocyclonucleosides, a class of compounds endowed with interesting antiviral activity, as it was evidenced for the first naturally occurring carbocyclonucleosides, aristeromycin and neplanocin [79,80]. ADA activity on carbocyclonucleosides is different with respect to nucleosides and, in fact, complete deamination of adenosine was accomplished in 2 h, while for aristeromycin, the carbocyclic analog of adenosine, 32 h, was required [81]. 2',3'-Dideoxyadenosine was completely deaminated at a similar rate to adenosine (2 h), while complete transformation

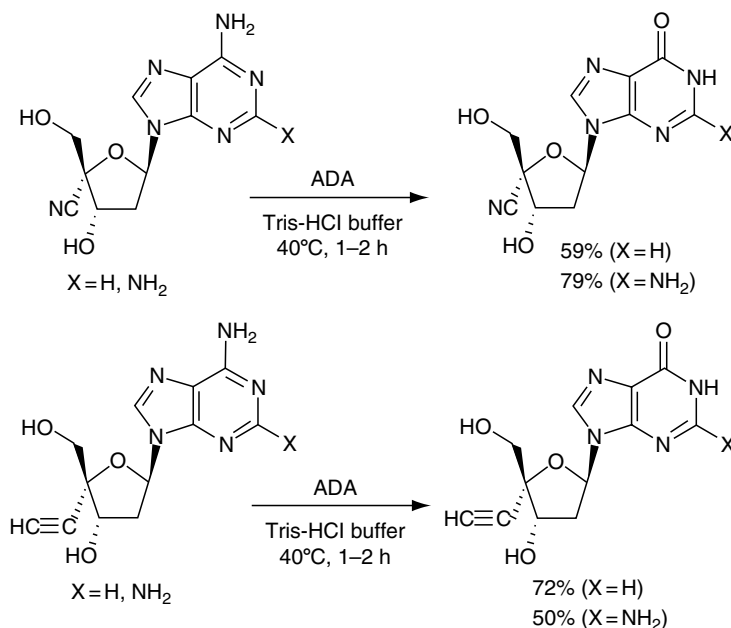


FIGURE 19.18 ADA-catalyzed deamination of 4'-substituted 2'-deoxypurine nucleosides.

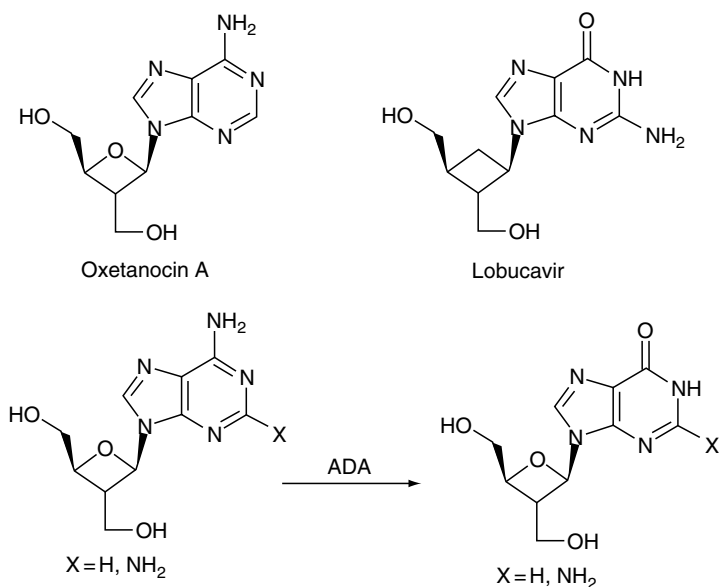


FIGURE 19.19 Structures of oxetanocin A, lobucavir, and ADA-catalyzed deamination of oxetanocin A and the 2-amino analog.

of 2',3'-dideoxyaristeromycin required 72 h (Figure 19.20). The utility of the chemoenzymatic approach for a preparative synthesis of carbocyclic inosines was also demonstrated.

Carbovir is a synthetic carbocyclonucleoside that found application as a chemotherapeutic agent for the treatment of AIDS and the selective inhibition of HIV resides in the (–) enantiomer [82]. (–)-Carbovir could be synthesized from structurally related (–)-aristeromycin

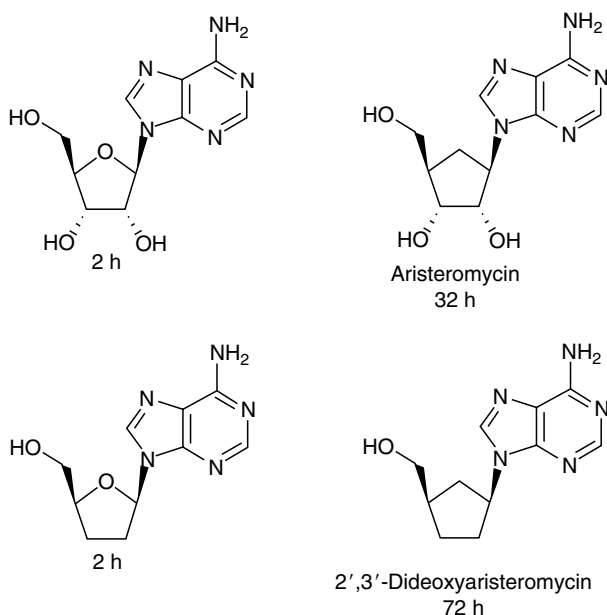


FIGURE 19.20 Time for complete ADA-catalyzed deamination of nucleosides and carbocyclonucleosides.

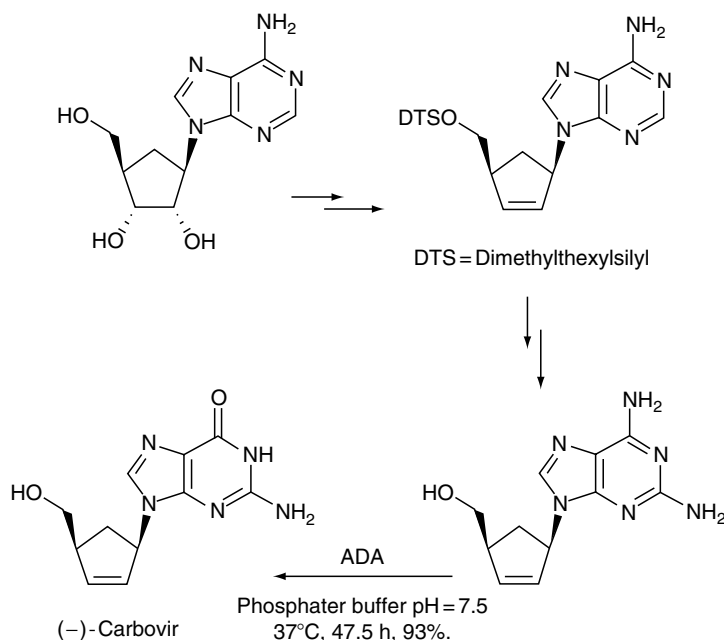


FIGURE 19.21 Chemoenzymatic synthesis of (-)-carbovir.

[83] and the guanine system was enzymatically prepared by quantitative deamination of 2,6-diaminopurine derivative in the presence of ADA (Figure 19.21).

19.11 ADA-ASSISTED CHEMOENZYMATIC SYNTHESIS OF ANTIVIRAL ACYCLONUCLEOSIDES

Acyclic analogs of nucleosides (acyclonucleosides) often present important antiviral activity, as shown by representative compounds such as acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine and ganciclovir, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine, that are at present well-established antiherpetic agents [84,85]. A few structurally related acyclonucleosides such as penciclovir [86], or its prodrug famciclovir [87] were introduced as antiviral agents during the past years (Figure 19.22).

In the above acyclonucleosides, the purine system is constituted by a guanine alkylated at the nitrogen atom indicated as N⁹ and synthetic methods for the preparation of leading compounds acyclovir or ganciclovir and their prodrugs have already been reviewed [12]. The action of ADA on suitable precursors may constitute a chemoenzymatic approach to the synthesis of these antiviral agents, therefore extending the potentiality of ADA as a biocatalyst. As previously reported, 2,6-diaminopurine nucleosides can be deaminated in the presence of ADA to the corresponding guanosines and this biocatalytic approach was extended to 2-amino-6-chloro- or 2,6-diamino-9-[(2-hydroxyethoxy)methyl]purines [88]. Complete ADA-catalyzed deamination of above compounds was observed in phosphate buffer at room temperature (Figure 19.23), while for the quantitative transformation of a suitable 2,6-diaminopurine acyclonucleoside into ganciclovir an excess of ADA was needed (18 h, 25°C) [89]. Another synthesis of both acyclovir and ganciclovir relies on the hydrolytic defluorination catalyzed by ADA of a 6-fluoropurine derivative that took place quantitatively overnight at room temperature [90].

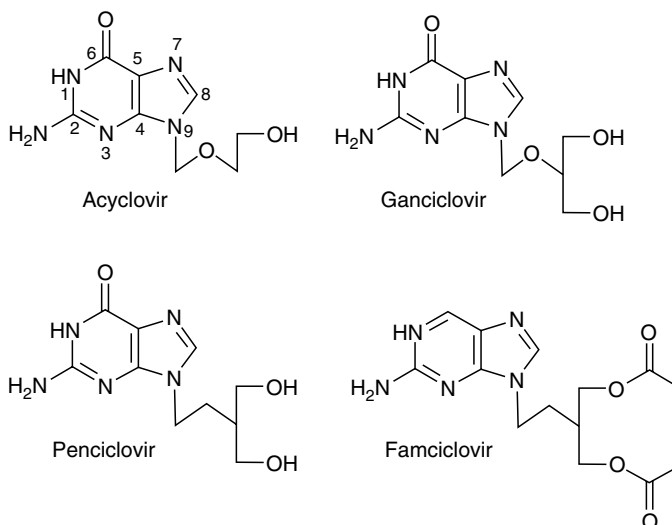


FIGURE 19.22 Structures of antiviral acyclonucleosides.

Unsaturated acyclonucleosides containing one or more double bonds that separate the nucleic base from an hydroxymethyl group are characterized by potent antiviral activity [91,92]. A few of the above compounds reported in Figure 19.24 are also substrates of ADA [92–97] and, as a general feature, the (*E*)-isomer reacts much faster than the (*Z*)-analog.

The same stereopreference has been shown by the enzyme for 2-[(hydroxymethyl)cyclopropylidene]methyladenine (Figure 19.25), characterized by a broad-spectrum antiviral activity [97]. ADA-catalyzed deamination proceeds more efficiently on the (*E*)-isomer and this can contribute to explain the higher activity of the (*Z*)-isomer (Synadenol).

19.12 AMPDA-ASSISTED SYNTHESIS AND TRANSFORMATION OF ANTIVIRAL NUCLEOSIDES AND ANALOGS

Compared to ADA, application of AMPDA to biocatalytic transformation of nucleosides and related analogs has been studied only recently [43]. The broader activity of AMPDA vs.

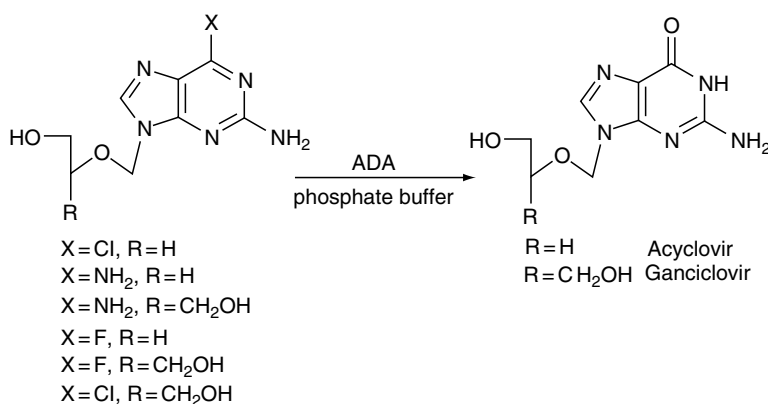


FIGURE 19.23 ADA-catalyzed deamination of precursors of acyclovir and ganciclovir.

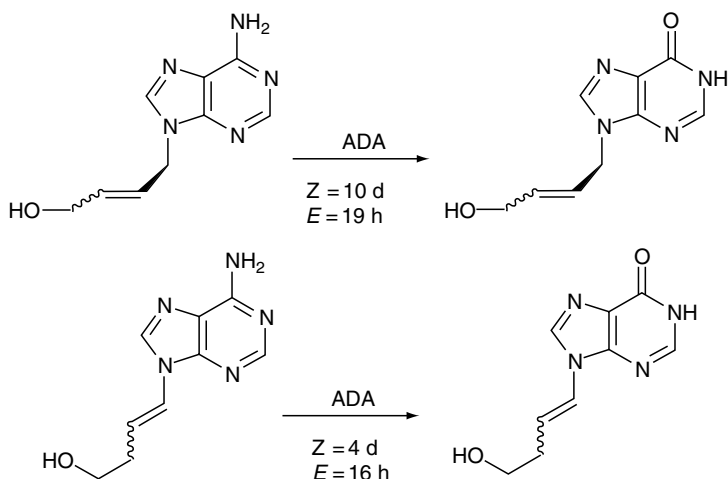


FIGURE 19.24 ADA-catalyzed selective deamination of unsaturated acyclonucleosides.

ADA, already evidenced in the above report, has been confirmed by more recent results [41]. Among compounds that were not substrate for ADA and were deaminated by AMPDA, (1*R*-*cis*)-bis(1-methylethyl) [2-[4-(2,6-diamino-9*H*-purin-9-yl)-2-cyclopenten-1-yl]ethyl]-1-phosphonate (Figure 19.26) was enzymatically converted into the corresponding guanosine derivative [43], i.e., carbovir phosphonate.

Another difference for AMPDA with respect to ADA has been observed in previous study on the antiviral activity of the 2-[(hydroxymethyl)cyclopropylidene]methyladenines [97]. In fact, the more active (*Z*)-isomer, Synadenol, that was only slowly deaminated by ADA, was efficiently transformed in the presence of AMPDA (>95%, 4.5 h, Figure 19.27).

19.13 STEREOSELECTIVITY OF DEAMINATING ENZYMES

The chemical synthesis of nucleosides invariably yields isomeric mixtures and the stereochemical specificity of deaminating enzymes (ADA and AMPDA) may represent an additional advantage of the biocatalysts. Therefore, applications of the enzymes might be specially appreciated to resolve problems of enantio- and diastereoselectivity in asymmetric synthesis of nucleosides. The stereochemical preference of ADA toward different isomers of synthetic nucleosides can be evidenced through the examples that will now be examined and discussed. AMPDA-catalyzed stereoselection of isomeric nucleosides or analogs will be also described

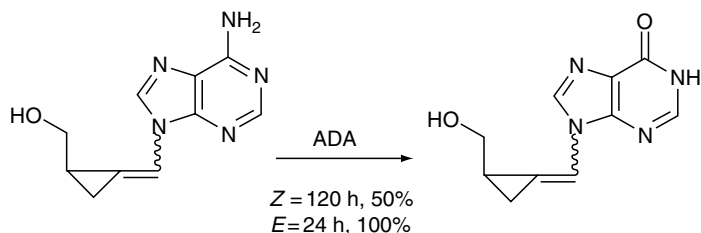


FIGURE 19.25 ADA-catalyzed selective deamination of (*E*)/(*Z*)-2-[(hydroxymethyl)cyclopropylidene]-methyladenine.

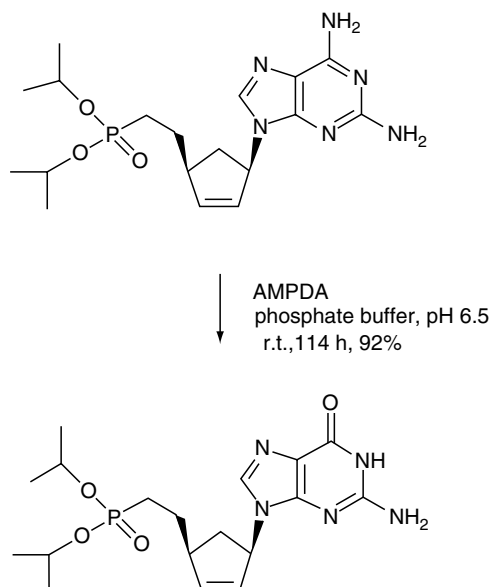


FIGURE 19.26 AMPDA-catalyzed deamination of a carbocyclonucleoside phosphonate.

but examples are rather limited in number, as the application of this enzyme in nucleoside chemistry has been only recently reported.

19.14 RESOLUTION OF SYNTHETIC NUCLEOSIDES AND ANALOGS

Since the introduction of Lamivudine (β -L-2',3'-dideoxy-3'-thiacytidine), there has been a considerable synthetic effort directed to the preparation of β -L-nucleosides as specific antiviral agents [98]. Based on the consideration that ADA is able to catalyze the deamination of natural nucleosides, it was expected that L-nucleosides should not be substrates of the enzyme or would react much slower than the naturally occurring D-isomer. This has been verified by kinetic studies on β -D- and L-2',3'-dideoxyadenosines [99] or D- and L-2',3'-dideoxy-2',3'-*endo*-methylene nucleosides, that were synthesized as potential antiviral agents [100]. Specifically, in the presence of ADA from calf intestinal mucosa and compared with adenosine ($t_{1/2} = 30$ s), the D-2',3'-dideoxy-2',3'-*endo*-methylene nucleoside was a good substrate ($t_{1/2} = 90$ s), while the L-isomer reacted slower ($t_{1/2} = 22$ h) (Figure 19.28).

Synthesis of carbocyclic analogs of natural nucleosides has to rely mainly, if not exclusively, on chemical methods that afford isomeric mixtures from which each stereoisomer has

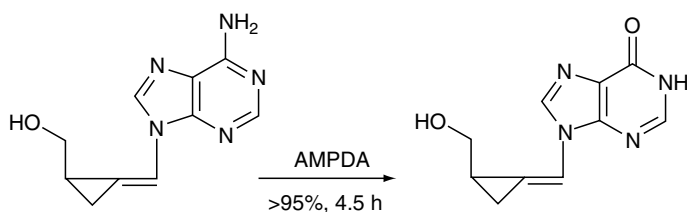


FIGURE 19.27 Selective deamination of (Z)-2-[(hydroxymethyl)cyclopropylidene]methyladenine by AMPDA.

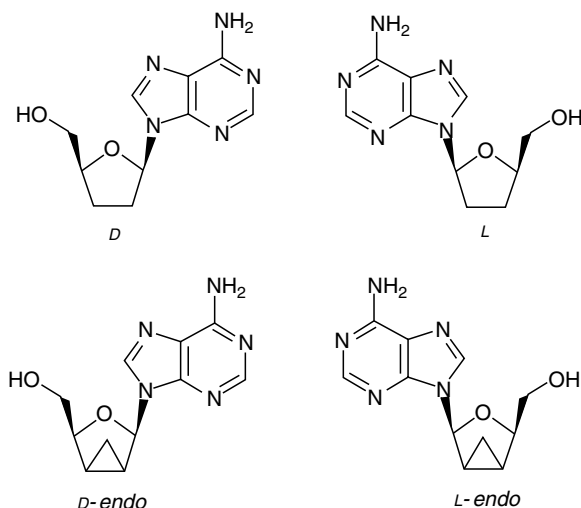


FIGURE 19.28 Structures of D- and L-modified adenosines, substrates for D-selective ADA-catalyzed deamination.

to be separated. Secrist and coworkers have described the first resolution of a racemic mixture of synthetic carbocyclic nucleosides [101]. Specifically, (\pm)-aristeromycin was resolved by ADA that deaminated the (–)-isomer into the corresponding hypoxanthine derivative (3 h, room temperature), so that the stereochemically pure unnatural (+)-aristeromycin could be recovered (Figure 19.29).

The same authors report another interesting application of ADA-catalyzed deamination for the resolution of carbocyclic 2'-deoxyguanosine starting from the racemic 2,6-diaminopurine analog [101]. The enzyme and substrate ratio and the temperature of the reaction played an essential role, since the unreacted (–)-compound was transformed into (–)-guanine derivative using ADA only at a lower temperature (37 instead of 50°C), but raising the enzyme and substrate ratio (from 0.5 to 4 U/ μ mol) (Figure 19.30).

Analogously, the same synthetic strategy was applied to obtain both enantiomers of carbovir starting from 9-(4-hydroxymethylcyclopent-2-enyl)-9H-purine-2,6-diamine that was deaminated to (–)-carbovir at 25°C in 72 h. Transformation of the unreacted (+)-diamino compound under different experimental conditions (37°C, 2 d) allowed the preparation of (+)-carbovir that was shown to be much less active as HSV-1 inhibitor (Figure 19.31) [82].

The resolution of racemic 2-amino-6-chloro pyrazolopyrimidine bound to a fluorinated cyclopentane moiety (Figure 19.32) is a further example of ADA versatility [102]. A research

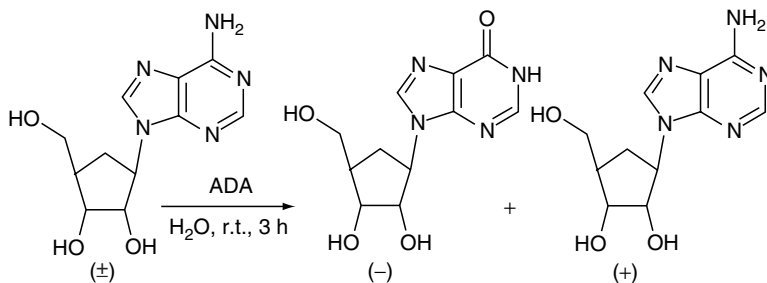


FIGURE 19.29 ADA-catalyzed resolution of synthetic racemic aristeromycin.

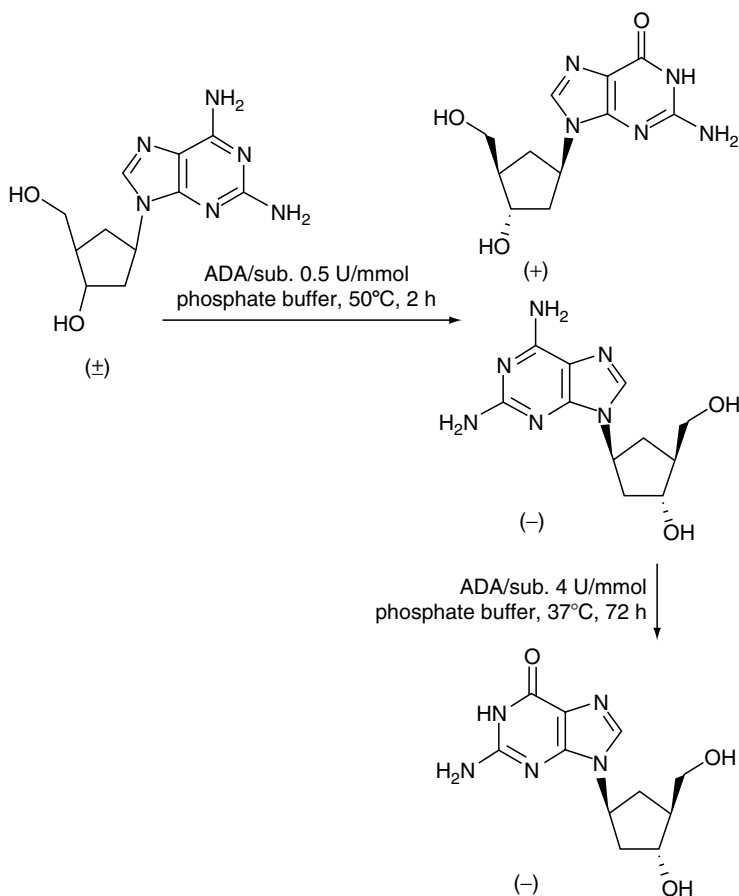


FIGURE 19.30 ADA-catalyzed selective deamination of a synthetic 2,6-diaminopurine carbocyclo-nucleoside.

program has been developed in recent years aimed to the synthesis and biological evaluation of conformationally restricted carbocyclic nucleosides, as exemplified by one of the latest reports [103]. Within this line of research, a racemic cyclopropyl analog of neplanocin (Figure 19.32) has been synthesized and an anti-HIV screening indicated that this adenosine analog possessed a level of activity that could be better evaluated by preparing individual enantiomers [104]. The resolution of the synthetic racemic mixture was successfully achieved by an ADA-catalyzed deamination.

Zemlicka and coworkers have prepared a series of antiviral acyclonucleosides characterized by the presence of unsaturations and cyclopropane ring in the acyclic moiety of the structure [105]. The compound containing the cyclopropyl moiety directly bound to adenine, 9-[(2-hydroxyethylidene)cyclopropyl]adenine, was incubated as a mixture (*E*)/(*Z*) with ADA. After 45 h at room temperature, 82% of the (*E*)-isomer was deaminated to the hypoxanthine analog, while the (*Z*)-isomer remained essentially unchanged (Figure 19.33) [106].

The stereopreference exhibited by ADA (Figure 19.33) is in agreement with the observation on single isomers of similar compounds reported by the same authors [97] and illustrated in Figure 19.25. It should also be recalled that (*Z*)-2-[(hydroxymethyl)cyclopropylidene]methyladenine has been deaminated in the presence of AMPDA, as shown in Figure 19.27 [97].

R,S-Adenallene is a strong inhibitor of the replication and cytopathic effect of HIV-1 and HIV-2 [107], the antiretroviral activity being higher for *R*-adenallene [108]. For the study of

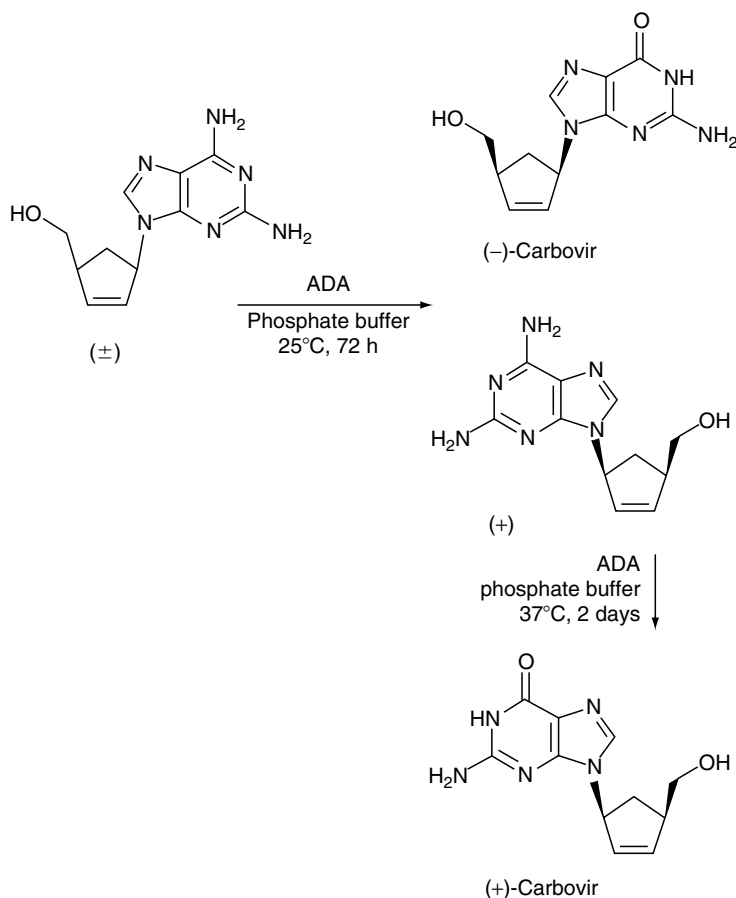


FIGURE 19.31 ADA-catalyzed selective deamination of 2,6-diaminocarbocyclonucleosides.

single-isomer action on enzymes of nucleic acid metabolism, *R*- and *S*-adenallene phosphates were required. *R,S*-Adenallene 4'-phosphate is deaminated with AMPDA with no enantioselectivity (Figure 19.34), whereas a partial resolution was achieved by slow enzymatic dephosphorylation catalyzed by 5'-nucleotidase (*R* 26%; *S* 54%) [109].

Other carbocyclonucleosides containing bicyclic systems characterized by small rings as spiroheptane and spiropentane mimics of nucleosides were studied as potential antiviral agents [110,111]. The spiroheptane analog of adenallene with axial dissymmetry was not

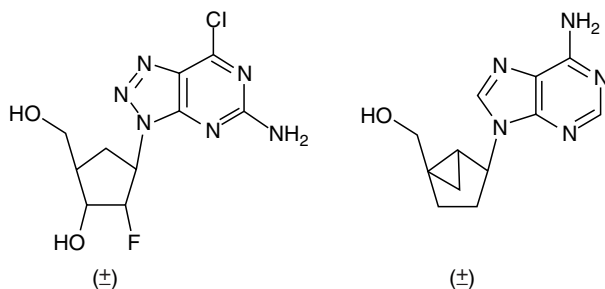


FIGURE 19.32 Racemic carbocyclic substrates resolved by ADA-catalyzed deamination.

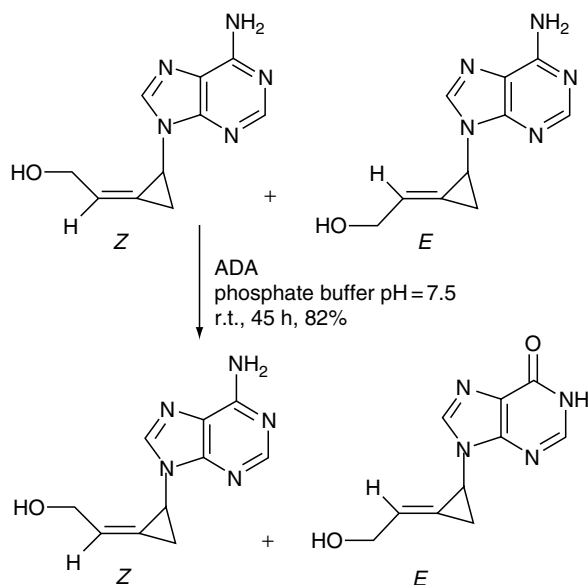


FIGURE 19.33 ADA-catalyzed selective deamination of (*E*)-9-[(2-hydroxyethylidene)-cyclopropyl]adenine.

substrate for ADA [110]. Among the synthesized stereoisomers of spiropentane mimics (Figure 19.35), only the *medial-anti*-isomer was slowly transformed by the deaminating enzyme (activity expressed as $t_{1/2} > 120$ h) [111].

19.15 DIASTEREOSELECTIVITY OF DEAMINATING ENZYMES

Finally, a particular type of diastereoselectivity was revealed for the enzymatic deamination catalyzed by ADA when a methyl group was introduced in the position 5' of modified adenosine and related analogs. An additional stereogenic center is generated at that position

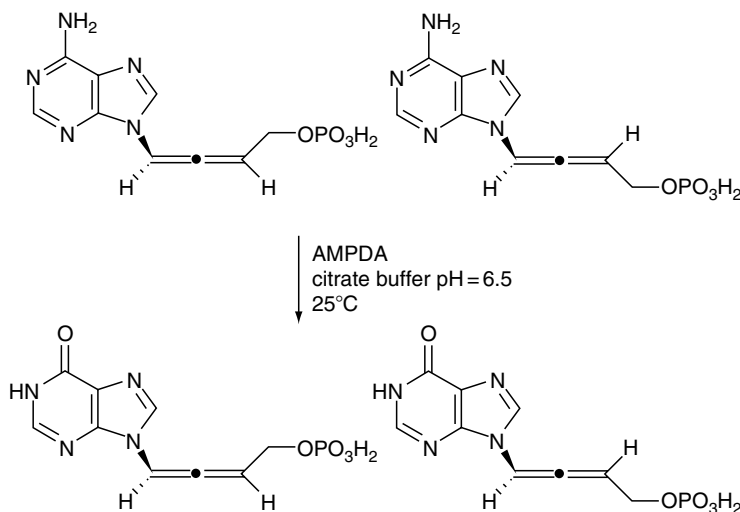


FIGURE 19.34 AMPDA-catalyzed nonselective deamination of *R,S*-adenallene-4'-phosphate.

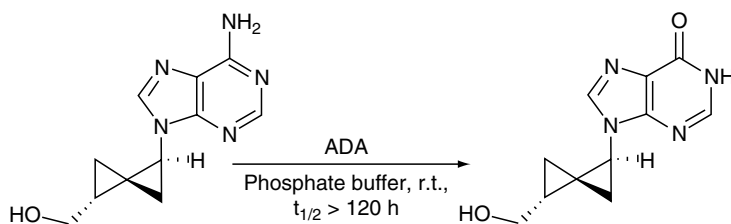


FIGURE 19.35 ADA-catalyzed deamination of *medial-anti*-spiropentane acyclonucleoside.

and the presence of a hydroxy group in the newly formed secondary alcohol is still compatible with the activity of the enzyme, that stereoselectively deaminates the 5'*S*-isomer [112]. Other studies on the ADA- and AMPDA-catalyzed selective deamination of a few modified adenosines bearing 5'-alkyl substitutions have also been recently reported [113].

An example of such stereoselectivity had been reported for the carbocyclonucleoside neplanocin A, once a methyl group was introduced at 6'-position. After separation of individual isomers by preparative HPLC, for 6'*R*-methyl neplanocin a significant antiviral activity was demonstrated, while 6'*S*-isomer was inactive [114]. When the synthetic diastereomeric mixture was submitted to the action of ADA, a selective hydrolysis of 6'*S*-methyl neplanocin to the corresponding hypoxanthine analog was observed and the active 6'*R*-isomer could be recovered (Figure 19.36) [115].

19.16 CONCLUDING REMARKS

The synthesis of modified purine nucleosides and related systems that present antiviral activity may rely on a vast array of selective chemical and biocatalytic procedures. Adenosine

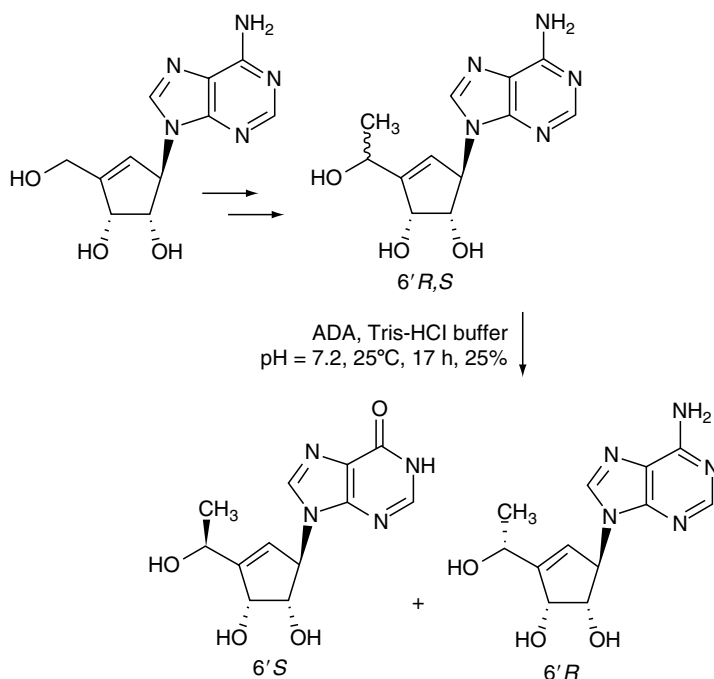


FIGURE 19.36 Diastereoselectivity of ADA-catalyzed deamination of 6'*R,S*-methyl neplanocin.

or adenylylase (ADA or AMPDA) are hydrolytic deaminating enzymes of the purine recycling or salvatage pathway that selectively converts 6-substituted purine nucleosides into the corresponding 6-oxo analogs. Both enzymes can accept substrates with a good degree of structural variations either in the base ring or in the furanose moiety. For this reason, they can be considered as valuable biocatalysts for application to synthetic chemistry of nucleosides and related carbocyclic or acyclic analogs. First applications of ADA date back to nearly 40 years ago and the well-established knowledge about its structure and mechanism makes this enzyme a relatively well-defined biocatalyst. Several examples of chemoenzymatic syntheses where ADA is required for selective transformation are already available. The substrates are nucleosides modified in the furanose and/or purine portion, carbocyclic or acyclic nucleosides and in the present review selected examples of antiviral compounds have been reported. Although AMPDA is commercially utilized for the synthesis of inosine monophosphate, the enzyme is less studied than ADA and only recent works have disclosed its capability. AMPDA appears to be less selective toward the substrate structure and is capable to offer a broader range of applications, thus further expanding potentiality of deaminating enzymes in the synthesis of modified nucleosides and analogs.

REFERENCES

1. Cheson, B.D., Keating, M.J., and Plunkett, W., (Eds.), *Nucleoside Analogs in Cancer Therapy*, Marcel Dekker, New York, 1997.
2. Galmarini, C.M., Mackey, J.R., and Dumontet, C., Nucleoside analogues and nucleobases in cancer treatment, *Lancet Oncol.*, 3(7), 415–424, 2002.
3. Marquez, V.E., Design, synthesis, and antiviral activity of nucleoside and nucleotide analogs, *ACS Symp. Ser.*, 401, 140–155, 1989.
4. Mackman, R.L. and Cihlar, T., Prodrug strategies in the design of nucleoside and nucleotide antiviral therapeutics, *Annu. Rep. Med. Chem.*, 39, 305–321, 2004.
5. Jonckheere, H., Annè, J., and De Clercq, E., The HIV-1 reverse transcription (RT) process as target for RT inhibitors, *Med. Res. Rev.*, 20(2), 129–154, 2000.
6. Macchi, B. and Mastino, A., Pharmacological and biological aspects of basic research on nucleoside-based reverse transcriptase inhibitors, *Pharmacol. Res.*, 46(6), 473–482, 2002.
7. Kusaka, T., Yamamoto, H., Shibata, M., et al., *Streptomyces citricolor* nov. sp. and a new antibiotic, aristeromycin, *J. Antibiot.*, 21(4), 255–263, 1968.
8. Yaginuma, S., Muto, N., Tsujino, M., et al., Studies on neplanocin A, new antitumor antibiotic. I. Producing organism, isolation and characterization, *J. Antibiot.*, 34(4), 359–366, 1981.
9. Borthwick, A.D. and Biggadike, K., Synthesis of chiral carbocyclic nucleosides, *Tetrahedron*, 48(4), 517–623, 1992.
10. Agrofoglio, L., Suhas, E., Farese, A., et al., Synthesis of carbocyclic nucleosides, *Tetrahedron*, 50(36), 10611–10670, 1994.
11. Crimmins, M.T., New developments in the enantioselective synthesis of cyclopentyl carbocyclic nucleosides, *Tetrahedron*, 54(32), 9229–9272, 1998.
12. Gao, H. and Mitra, A.K., Synthesis of acyclovir, ganciclovir and their prodrugs: a review, *Synthesis*, 3, 329–351, 2000.
13. Prasad, A.K. and Wengel, J., Enzyme-mediated protecting group chemistry on the hydroxyl groups of nucleosides, *Nucleosides Nucleotides*, 15(7–8), 1347–1359, 1996.
14. Prasad, A.K., Tripathi, S., and Parmar, V.S., Nucleoside synthesis mediated by glycosyl transferring enzymes, *Bioorg. Chem.*, 27(2), 135–154, 1999.
15. Ferrero, M. and Gotor, V., Biocatalytic selective modifications of conventional nucleosides, carbocyclic nucleosides, and C-nucleosides, *Chem. Rev.*, 100(12), 4319–4347, 2000.
16. Hanrahan, J.R. and Hutchinson, D.W., The enzymatic synthesis of antiviral agents, *J. Biotechnol.*, 23(2), 193–210, 1992.

17. Zielke, C.L. and Suelter, C.H., Purine, purine nucleoside, and purine nucleotide aminohydrolases, in *Enzymes*, 3rd ed., vol. 4, Boyer, P.D., Ed., Academic Press, New York, 1971, pp. 47–78.
18. Santaniello, E., Ciuffreda, P., and Alessandrini, L., Synthesis of modified purine nucleosides and related compounds mediated by adenosine deaminase (ADA) and adenylate deaminase (AMPDA), *Synthesis*, 4, 509–526, 2005.
19. Cristalli, G., Costanzi, S., Lambertucci, C., et al., Adenosine deaminase: functional implications and different classes of inhibitors, *Med. Res. Rev.*, 21(2), 105–128, 2001.
20. Lushchak, V.I., Functional role and properties of AMP-deaminase, *Biochemistry (Mosc)*, 61(2), 143–154, 1996.
21. Samejima, H., Kimura, K., Noguchi, S., et al., Production of 5'-mononucleotides using immobilized 5'-phosphodiesterase and 5'-AMP deaminase, in *Enzyme Engineering*, vol. 3, Pye, E.K. and Weetall, H.H., Eds., Plenum Press, New York, 1978, pp. 469–475.
22. Jones, J.B. and Beck, J.F., Asymmetric syntheses and resolutions using enzymes, in *Application of Biochemical Systems in Organic Chemistry*, Jones, J.B., Sih, C.J., and Perlman, D., Eds., Wiley, New York, 1976, part I, pp. 107–401.
23. Wilson, D.K., Rudolph, F.B., and Quiocho, F.A., Atomic-structure of adenosine-deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations, *Science*, 252(5010), 1278–1284, 1991.
24. Sharff, A.J., Wilson, D.K., Chang, Z., et al., Refined 2.5 Å structure of murine adenosine deaminase at pH 6.0, *J. Mol. Biol.*, 226(4), 917–921, 1992.
25. Wang, Z. and Quiocho, F.A., Complexes of adenosine deaminase with two potent inhibitors: x-ray structures in four independent molecules at pH of maximum activity, *Biochemistry*, 37(23), 8314–8324, 1998.
26. Kinoshita, T., Nishio, N., Nakanishi, I., et al., Structure of bovine adenosine deaminase complexed with 6-hydroxy-1,6-dihydropurine riboside, *Acta Cryst.*, D59, 299–303, 2003.
27. Frieden, C., Kurz, L.C., and Gilbert, H.R., Adenosine deaminase and adenylate deaminase: comparative kinetic studies with transition state and ground state analogue inhibitors, *Biochemistry*, 19(23), 5303–5309, 1980.
28. Bolen, D.W. and Fisher, J.R., Kinetic properties of adenosine deaminase in mixed aqueous solvents, *Biochemistry*, 8(11), 4239–4246, 1969.
29. Cory, J.G. and Suhadolnik, R.J., Dechloronase activity of adenosine deaminase, *Biochemistry*, 4(9), 1733–1735, 1965.
30. Wolfenden, R., Enzymatic hydrolysis of 6-substituents on purine ribosides, *J. Am. Chem. Soc.*, 88(13), 3157–3158, 1966.
31. Chassy, B.M. and Suhadolnik, R.J., Adenosine aminohydrolase. Binding and hydrolysis of 2- and 6-substituted purine ribonucleosides and 9-substituted adenine nucleosides, *J. Biol. Chem.*, 242(16), 3655–3658, 1967.
32. Maguire, M.H. and Sim, M.K., Studies on adenosine deaminase. 2. Specificity and mechanism of action of bovine placental adenosine deaminase, *Eur. J. Biochem.*, 23(1), 22–29, 1971.
33. Wanner, M.J., Deghati, P.Y.F., Rodenko, B., et al., New nucleoside analogs, synthesis, and biological properties, *Pure Appl. Chem.*, 72(9), 1705–1708, 2000.
34. Cory, J.G. and Suhadolnik, R.J., Structural requirements of nucleosides for binding by adenosine deaminase, *Biochemistry*, 4(9), 1729–1732, 1965.
35. Bloch, A., Robins, M.J., and McCarthy, J.R., Jr., The role of the 5'-hydroxyl group of adenosine in determining substrate specificity for adenosine deaminase, *J. Med. Chem.*, 10(5), 908–912, 1967.
36. Maury, G., Daiboun, T., Elalaoui, A., et al., Inhibition and substrate specificity of adenosine deaminase. Interaction with 2',3'- and/or 5'-substituted adenine nucleoside derivatives, *Nucleosides Nucleotides*, 10(8), 1677–1692, 1991.
37. Chae, W.-G., Chan, T.C.K., and Chang, C., Facile synthesis of 5'-deoxy- and 2',5'-dideoxy-6-thiopurine nucleosides by nucleoside phosphorylases, *Tetrahedron*, 54(30), 8661–8670, 1998.
38. Wnuk, S.F., Stoeckler, J.D., and Robins, M.J., Nucleic acid related compounds. 82. Conversions of adenosine to inosine 5'-thioether derivatives with *Aspergillus oryzae* adenosine deaminase or alkyl nitrites. Substrate and inhibitory activities of inosine 5'-thioether derivatives with purine nucleoside phosphorylase, *Nucleosides Nucleotides*, 13(1–3), 389–403, 1994.

39. Ciuffreda, P., Loseto, A., Alessandrini, L., et al., Adenylate deaminase (5'-adenylic acid deaminase, AMPDA)-catalyzed deamination of 5'-deoxy-5'-substituted and 5'-protected adenosines: a comparison with the catalytic activity of adenosine deaminase (ADA), *Eur. J. Org. Chem.*, (24), 4748–4751, 2003.
40. Ciuffreda, P., Casati, S., and Santaniello, E., The action of adenosine deaminase (EC 3.5.4.4.) on adenosine and deoxyadenosine acetates: the crucial role of the 5'-hydroxy group for the enzyme activity, *Tetrahedron*, 56(20), 3239–3243, 2000.
41. Ciuffreda, P., Loseto, A., and Santaniello, E., Deamination of 5'-substituted-2', 3'-isopropylidene adenosine derivatives catalyzed by adenosine deaminase (ADA, EC 3.5.4.4) and complementary enzymatic biotransformations catalyzed by adenylate deaminase (AMPDA, EC 3.5.4.6): a viable route for the preparation of 5'-substituted inosine derivatives, *Tetrahedron*, 58(29), 5767–5771, 2002.
42. Robins, M.J., Zou, R., Hansske, F., et al., Synthesis of sugar-modified 2,6-diaminopurine and guanine nucleosides from guanosine via transformations of 2-aminoadenosine and enzymatic deamination with adenosine deaminase, *Can. J. Chem.*, 75(6), 762–767, 1997.
43. Margolin, A.L., Borcherdig, D.R., Wolf-Kugel, D., et al., AMP-deaminase as a novel practical catalyst in the synthesis of 6-oxopurine ribosides and their analogs, *J. Org. Chem.*, 59(24), 7214–7218, 1994.
44. Mitsuya, H. and Broder, S., Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides, *Proc. Natl. Acad. Sci. USA*, 83(6), 1911–1915, 1986.
45. Shelton, M.J., O'Donnell, A.M., Morse, G.D., *Didanosine. Annal. Pharmacother.*, 26(5), 660–670, 1992.
46. Bhat, V., Stocker, E., and Ugarkar, B.G., A new synthesis of 2',3'-dideoxyinosine, *Synth. Commun.*, 22(10), 1481–1486, 1992.
47. Chu, C.K., Bhadti, V.S., Doboszewski, B., et al., General syntheses of 2',3'-dideoxynucleosides and 2',3'-didehydro-2',3'-dideoxynucleosides, *J. Org. Chem.*, 70(14), 2217–2225, 1989.
48. Webb, R.R., Wos, J.A., Martin, J.C., et al., Synthesis of 2',3'-dideoxyinosine, *Nucleosides Nucleotides*, 7(2), 147–155, 1988.
49. Ciuffreda, P., Casati, S., and Santaniello, E., Lipase-catalyzed protection of the hydroxy groups of the nucleosides inosine and 2'-deoxyinosine: a new chemo-enzymatic synthesis of the antiviral drug 2',3'-dideoxyinosine, *Bioorg. Med. Chem. Lett.*, 9(11), 1577–1582, 1999.
50. Robins, M.J., Wilson, J.S., and Hansske, F., Nucleic acid related compounds. 42. A general procedure for the efficient deoxygenation of secondary alcohols. Regiospecific and stereoselective conversion of ribonucleosides to 2'-deoxynucleosides, *J. Am. Chem. Soc.*, 105(12), 4059–4065, 1983.
51. Buenger, G.S. and Nair, V., Dideoxygenated purine nucleosides substituted at the 8-position: chemical synthesis and stability, *Synthesis*, 10, 962–966, 1990.
52. Ikehara, M. and Fukui, T., Studies of nucleosides and nucleotides. LVIII. Deamination of adenosine analogs with calf intestine adenosine deaminase, *Biochim. Biophys. Acta*, 338(2), 512–519, 1974.
53. Seela, F. and Kaiser, K., 8-Aza-7-deaza-2',3'-dideoxyadenosine: synthesis and conversion into allopurinol 2',3'-dideoxyribofuranoside, *Chem. Pharm. Bull.*, 36(10), 4153–4156, 1988.
54. Baer, H.P., Drummond, G.I., and Duncan, E.L., Formation and deamination of adenosine by cardiac muscle enzymes, *Mol. Pharmacol.*, 2(1), 67–76, 1966.
55. Mengel, R. and Muhs, W., Nucleoside transformations. V. Transformation of guanosine into 2'-deoxy-, 3'-deoxy-, 2',3'-anhydro- and xylo-guanosine, *Chem. Ber.*, 112(2), 625–639, 1979.
56. Jain, T.C., Jenkins, D.I., Russell, A.F., et al., Reactions of 2-acyloxyisobutryl halides with nucleosides. IV. A facile synthesis of 2',3'-unsaturated nucleosides using chromous acetate, *J. Org. Chem.*, 39(1), 30–38, 1974.
57. Suzuki, S., Lee, B., Luo, W., et al., Inhibition of duck hepatitis B virus replication by purine 2',3'-dideoxynucleosides, *Biochem. Biophys. Res. Commun.*, 156(3), 1144–1151, 1988.
58. Lee, B., Luo, W.-X., Suzuki, S., et al., *In vitro* and *in vivo* comparison of the abilities of purine and pyrimidine 2',3'-dideoxynucleosides to inhibit duck hepadnavirus, *Antimicrob. Agents Chemother.*, 33(3), 336–339, 1989.

59. Balzarini, J., Pauwels, R., Baba, M., et al., The 2',3'-dideoxyriboside of 2,6-diaminopurine selectively inhibits human immunodeficiency virus (HIV) replication *in vitro*, *Biochem. Biophys. Res. Commun.*, 145(1), 269–276, 1987.
60. Balzarini, J., Robins, M.J., Zou, R.M., et al., The 2',3'-dideoxyriboside of 2,6-diaminopurine and its 2',3'-didehydro derivative inhibit the deamination of 2',3'-dideoxyadenosine, an inhibitor of human immunodeficiency virus (HIV) replication, *Biochem. Biophys. Res. Commun.*, 145(1), 277–283, 1987.
61. Pauwels, R., Baba, M., Balzarini, J., et al., Investigations on the anti-HIV activity of 2',3'-dideoxyadenosine analogues with modifications in either the pentose or purine moiety. Potent and selective anti-HIV activity of 2,6-diaminopurine 2',3'-dideoxyriboside, *Biochem. Pharmacol.*, 37(7), 1317–1325, 1988.
62. Nair, V. and Sells, T.B., Enzymatic synthesis of 2',3'-dideoxyguanosine, *Synlett*, 10, 753–754, 1991.
63. Robins, M.J., Wilson, J.S., Madej, D., et al., Nucleic acid related compounds. 114. Synthesis of 2,6-(disubstituted)purine 2',3'-dideoxynucleosides and selected cytotoxic, anti-hepatitis B, and adenosine deaminase substrate activities, *J. Heterocycl. Chem.*, 38(6), 1297–1306, 2001.
64. Marquez, V.E., Tseng, C.K.H., Mitsuya, H., et al., Acid-stable 2'-fluoro purine dideoxynucleosides as active agents against HIV, *J. Med. Chem.*, 33(3), 978–985, 1990.
65. Ford, H., Jr., Siddiqui, M.A., Driscoll, J.S., et al., Lipophilic, acid-stable, adenosine deaminase-activated anti-HIV prodrugs for central nervous system delivery. 2. 6-Halo and 6-alkoxy prodrugs of 2'- β -fluoro-2',3'-dideoxyinosine, *J. Med. Chem.*, 38(7), 1189–1195, 1995.
66. Driscoll, J.S., Siddiqui, M.A., Ford H., Jr., et al., Lipophilic, acid-stable, adenosine deaminase-activated anti-HIV prodrugs for central nervous system delivery. 3. 6-Amino prodrugs of 2'- β -fluoro-2',3'-dideoxyinosine, *J. Med. Chem.*, 39(8), 1619–1625, 1996.
67. Shortnacy-Fowler, A.T., Tiwari, K.N., Montgomery, J.A., et al., Synthesis and biological activity of 2'-fluoro-D-arabinofuranosylpyrazolo[3,4-*d*]pyridine nucleosides, *Helv. Chim. Acta*, 82(12), 2240–2245, 1999.
68. Jung, P.M.J., Burger, A., and Biellmann, J.-F., Diastereofacial selective addition of ethynylcerium reagent and Barton-McCombie reaction as the key steps for the synthesis of C-3'-ethynylribonucleosides and of C-3'-ethynyl-2'-deoxyribonucleosides, *J. Org. Chem.*, 62(24), 8309–8314, 1997.
69. Tritesch, D., Jung, P.M., Burger, A., et al., 3'- β -ethynyl and 2'-deoxy-3'- β -ethynyl adenosines: first 3'- β -branched-adenosines substrates of adenosine deaminase, *Bioorg. Med. Chem. Lett.*, 10(2), 139–141, 2000.
70. Ohnishi, H. and Mitsuya, H., 4'-C-substituted-2'-deoxynucleosides: a family of antiretroviral agents which are potent against drug-resistant HIV variants, *Curr. Drug Targets Infect. Disord.*, 1(1), 1–10, 2001.
71. Nomura, M., Shuto, S., Tanaka, M., et al., Nucleosides and nucleotides. 185. Synthesis and biological activities of 4' α -C-branched-chain sugar pyrimidine nucleosides, *J. Med. Chem.*, 42(15), 2901–2908, 1999.
72. Ohnishi, H., Kohgo, S., Kitano, K., et al. Syntheses of 4'-C-ethynyl- β -D-arabino- and 4'-C-ethynyl-2'-deoxy- β -D-ribo-pentofuranosylpyrimidines and -purines and evaluation of their anti-HIV activity, *J. Med. Chem.*, 43(23), 4516–4525, 2000.
73. Kodama, E.-I., Kohgo, S., Kitano, K., et al., 4'-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants *in vitro*, *Antimicrob. Agents Chemother.*, 45(5), 1539–1546, 2001.
74. Kohgo, S., Yamada, K., Kitano, K., et al., Design, efficient synthesis, and anti-HIV activity of 4'-C-cyano- and 4'-C-ethynyl-2'-deoxy purine nucleosides, *Nucleosides Nucleotides Nucleic Acids*, 23(4), 671–690, 2004.
75. Shimada, N., Hasegawa, S., Harada, T., et al., Oxetanocin, a novel nucleoside from bacteria, *J. Antibiot.*, 39(11), 1623–1625, 1986.
76. Hoshino, H., Shimizu, N., Shimada, N., et al., Inhibition of infectivity of human immunodeficiency virus by oxetanocin, *J. Antibiot.*, 40(7), 1077–1078, 1987.
77. Ireland, C., Leeson, P.A., and Castaner, J., Lobucavir, *Drugs Future*, 22(4), 359–370, 1997.
78. Shimada, N., Hasegawa, S., Saito, S., et al., Derivatives of oxetanocin: oxetanocins H, X, and G, and 2-aminooxetanocin A, *J. Antibiot.*, 40(12), 1788–1790, 1987.

79. Marquez, V.E. and Lim M.-I., Carbocyclic nucleosides, *Med. Res. Rev.*, 6(1), 1–40, 1986.
80. Marquez, V.E., Carbocyclic nucleosides, in *Advances in Antiviral Drug Design*, vol. 2, De Clercq, E., Ed., JAI Press, Greenwich, CT, 1996, pp. 89–146.
81. Gala, D. and Schumacher, D.P., Deamination of carbocyclic adenosine analogs with adenosine deaminase and its synthetic utility, *Synlett*, 1, 61–63, 1992.
82. Vince, R. and Brownell, J., Resolution of racemic carbovir and selective inhibition of human immunodeficiency virus by the (–) enantiomer, *Biochem. Biophys. Res. Commun.*, 168(3), 912–916, 1990.
83. Exall, A.M., Jones, M.F., Mo, C.-L., et al., Synthesis from (–)-aristeromycin and x-ray structure of (–)-carbovir, *J. Chem. Soc. Perkin Trans. I*, 10, 2467–2477, 1991.
84. Schaeffer, H.J., Beauchamp, L., de Miranda, P., et al., 9-(2-Hydroxyethoxymethyl) guanine activity against viruses of the herpes group, *Nature*, 272(5654), 583–585, 1978.
85. Martin, J.C., Dvorak, C.A., Smee, D.F., et al., 9-[(1,3-Dihydroxy-2-propoxymethyl)]guanine: a new potent and selective antiherpes agent, *J. Med. Chem.*, 26(5), 759–761, 1983.
86. Harnden, M.R., Jarvest, R.L., Bacon T.H., et al., Synthesis and antiviral activity of 9-[4-hydroxy-3-(hydroxymethyl)but-1-yl]purines, *J. Med. Chem.*, 30(9), 1636–1642, 1987.
87. Vere Hodge, R.A., Sutton, D., Boyd, M.R., et al., Selection of an oral prodrug (BRL 42810; famciclovir) for the antiherpesvirus agent BRL 39123 [9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine; penciclovir], *Antimicrob. Agents Chemother.*, 33(10), 1765–1773, 1989.
88. Robins, M.J. and Hatfield, P.W., Nucleic acid related compounds, 37, convenient and high-yield syntheses of *N*-[(2-hydroxyethoxy)methyl]heterocycles as “acyclic nucleoside” analogues, *Can. J. Chem.*, 60(5), 547–553, 1982.
89. Ogilvie, K.K., Nguyen-ba, N., Gillen, M.F., et al., Synthesis of a purine acyclonucleoside series having pronounced antiviral activity, The glyceropurines, *Can. J. Chem.*, 62(2), 241–252, 1984.
90. Kim, D.K., Kim, H.K., and Chae, Y.-B., Design and synthesis of 6-fluoropurine acyclonucleosides: potential prodrugs of acyclovir and ganciclovir, *Bioorg. Med. Chem. Lett.*, 4(11), 1309–1312, 1994.
91. Haines, D., Tseng, C., and Marquez, V.E., Synthesis and biological activity of unsaturated carbocyclic purine nucleoside analogues, *J. Med. Chem.*, 30(5), 943–947, 1987.
92. Phadtare, S. and Zemlicka, J., Nucleic acid derived allenols. Unusual analogs of nucleosides with antiretroviral activity, *J. Am. Chem. Soc.*, 111(15), 5925–5931, 1989.
93. Phadtare, S., Kessel, D., Corbett, T.H., et al., Unsaturated and carbocyclic nucleoside analogues: synthesis, antitumor and antiviral activity, *J. Med. Chem.*, 34(1), 421–429, 1991.
94. Xu, Z.-Q., Qiu, Y.-L., Chokekijchai, S., et al., Unsaturated acyclic analogues of 2′-deoxyadenosine and thymidine containing fluorine: synthesis and biological activity, *J. Med. Chem.*, 38(6), 875–882, 1995.
95. Phadtare, S. and Zemlicka, J., Synthesis of (Z)- and (E)-N⁹-(4-hydroxy-1-buten-1-yl)adenine. New unsaturated analogs of adenosine, *Tetrahedron Lett.*, 31(1), 43–46, 1990.
96. Phadtare, S. and Zemlicka, J., Synthesis and biological properties of 9-(trans-4-hydroxy-2-buten-1-yl)adenine and guanine: open-chain analogues of neplanocin A, *J. Med. Chem.*, 30(2), 437–440, 1987.
97. Qiu, Y.-L., Ksebati, M.B., Ptak, R.G., et al., (Z)- and (E)-2-(Hydroxymethyl) cyclopropylidene)methyladenine and -guanine. New nucleoside analogues with a broad-spectrum antiviral activity, *J. Med. Chem.*, 41(1), 10–23, 1998.
98. Standring, D.N., Bridges, E.G., Placidi, L., et al., Antiviral β-L-nucleosides specific for hepatitis B virus infection, *Antivir. Chem. Chemother.*, 12(Suppl. 1), 119–121, 2001.
99. Pelicano, H., Pierra, C., Eriksson, S., et al., Enzymatic properties of the unnatural β-L-enantiomers of 2′,3′-dideoxyadenosine and 2′,3′-didehydro-2′,3′-dideoxyadenosine, *J. Med. Chem.*, 40(24), 3969–3973, 1997.
100. Chun, B.K., Olgen, S., Hong, J.H., et al., Enantiomeric syntheses of conformationally restricted D- and L-2′,3′-dideoxy-2′,3′-endo-methylene nucleosides from carbohydrate chiral templates, *J. Org. Chem.*, 65(3), 685–693, 2000.
101. Secrist III, J.A., Montgomery, J.A., Shealy, Y.F., et al., Resolution of racemic carbocyclic analogues of purine nucleosides through the action of adenosine deaminase. Antiviral activity of the carbocyclic 2′-deoxyguanosine enantiomers, *J. Med. Chem.*, 30(4), 746–749, 1987.

102. Borthwich, A.D., Biggadike, K., and Kirk, B.E., et al., Preparation of cyclopentyl triazolopyrimidones as antivirals and pharmaceutical compositions containing them, *Eur. Pat. Appl.*, 0 410 660, 1991.
103. Moon, H.R., Ford, H., and Marquez, V.E., A remarkably simple chemicoenzymatic approach to structurally complex bicyclo[3.1.0]hexane carbocyclic nucleosides, *Org. Lett.*, 2(24), 3793–3796, 2000.
104. Rodriguez, J.B., Marquez, V.E., Nicklaus, M.C., et al., Conformationally locked nucleoside analogues. Synthesis of dideoxycarbocyclic nucleoside analogues structurally related to neplanocin C, *J. Med. Chem.*, 37(20), 3389–3399, 1994.
105. Zemlicka, J., Unusual analogues of nucleosides: chemistry and biological activity, in *Recent Advances in Nucleosides: Chemistry and Chemotherapy*, Chu, C.K., Ed., Elsevier Science, Amsterdam, 2002, pp. 327–357.
106. Qiu, Y.-L., Ksebati, M.B., and Zemlicka, J., Synthesis of (Z)- and (E)-9-[(2-hydroxyethylidene)-cyclopropyl]adenine. New methylenecyclopropane analogues of adenosine and their substrate activity for adenosine deaminase, *Nucleosides Nucleotides Nucleic Acids*, 19(1–2), 31–37, 2000.
107. Zemlicka, J., Allenols derived from nucleic acid bases: a new class of anti-HIV agents: chemistry and biological activity, in *Nucleosides and Nucleotides as Antitumor and Antiviral agents*, Chu, C.K. and Baker, D.C., Eds., Plenum, New York, 1993, pp. 73–100.
108. Megati, S., Goren, Z., Silverton, J.V., et al., (R)-(-)- and (S)-(+)-Adenallene: synthesis, absolute configuration, enantioselectivity of antiretroviral effect, and enzymic deamination, *J. Med. Chem.*, 35(22), 4089–4104, 1992.
109. Jones, B.C.N.M. and Zemlicka, J., R,S-Adenallene 4'-phosphate: substrate activity and enantioselectivity toward AMP deaminase and 5'-nucleotidase, *Biorg. Med. Chem. Lett.*, 5(15), 1633–1636, 1995.
110. Jones, B.C.N.M., Drach, J.C., Corbett, T.H., et al., (±)-N⁹-(2-(Hydroxymethyl) spiro[3.3]hept-6-yl) adenine. The first biologically active saturated analogue of adenallene with axial dissymmetry, *J. Org. Chem.*, 60(20), 6277–6280, 1995.
111. Guan, H.-P., Ksebati, M.B., Cheng, Y.-C., et al., Spiropentane mimics of nucleosides: analogues of 2'-deoxyadenosine and 2'-deoxyguanosine. Synthesis of all stereoisomers, isomeric assignment, and biological activity, *J. Org. Chem.*, 65(5), 1280–1290, 2000.
112. Ciuffreda, P., Loseto, A., and Santaniello, E., Stereoselective deamination of (5'RS)-5'-methyl-2',3'-isopropylidene adenosine catalyzed by adenosine deaminase: preparation of diastereomerically pure 5'-methyl adenosine and inosine derivatives, *Tetrahedron Asymmetry*, 13(3), 239–241, 2002.
113. Ciuffreda, P., Loseto, A., and Santaniello, E., Stereoselective adenylate deaminase (5'-adenylic acid deaminase, AMPDA)-catalyzed deamination of 5'-alkyl substituted adenosines: a comparison with the action of adenosine deaminase (ADA), *Tetrahedron Asymmetry*, 15(2), 203–206, 2004.
114. Shuto, S., Obara, T., Toriya, M., et al., New neplanocin analogues. 1. Synthesis of 6'-modified neplanocin A derivatives as broad-spectrum antiviral agents, *J. Med. Chem.*, 35(2), 324–331, 1992.
115. Shuto, S., Obara, T., Yaginuma, S., et al., New neplanocin analogues. IX. A practical preparation of (6'R)-6'-C-methylnepanocin A (RMNPA), a potent antiviral agent, and the determination of its 6'-configuration. Diastereoselective deamination by adenosine deaminase, *Chem. Pharm. Bull.*, 45(1), 138–142, 1997.

20 Microbial and Enzymatic Processes for the Production of Chiral Compounds

*Kohsuke Honda, Takeru Ishige, Michihiko Kataoka,
and Sakayu Shimizu*

CONTENTS

20.1	Introduction	529
20.2	A Novel Tool for Enzymatic Optical Resolution—Lactonase.....	530
20.2.1	What Is a Lactonase?.....	530
20.2.2	Optical Resolution of DL-Pantoyl Lactone by a Fungal Lactonase	530
20.2.2.1	Discovery of a Novel Lactonase	530
20.2.2.2	Properties of the Lactonase from <i>Fusarium Oxysporum</i>	531
20.2.2.3	Development of a Practical Resolution System	532
20.2.2.4	Recent Progress	533
20.2.3	Other Microbial Lactonases.....	533
20.2.3.1	L-Pantoyl Lactone Hydrolase from <i>Agrobacterium Tumefaciens</i>	533
20.2.3.2	Dihydrocoumarin Hydrolase from <i>Acinetobacter Calcoaceticus</i> ..	533
20.2.3.3	(<i>R</i>)- δ -Decanolactone Hydrolase from <i>Pseudomonas</i> sp. 3–1.....	533
20.3	Direct synthesis of Chiral Alcohols with Stereoselective Carbonyl Reductases	535
20.3.1	Methodological Advantages of Direct Synthesis	535
20.3.2	Diversity of Carbonyl Reductases.....	535
20.3.3	Cofactor Regenerating Systems	535
20.3.3.1	Single Enzyme Systems	535
20.3.3.2	Coupled Enzyme Systems	536
20.3.4	Practical Applications of Carbonyl Reductases	537
20.3.4.1	Production of 4-Chloro-3-Hydroxybutanoate Ethyl Ester through Alternative Processes.....	537
20.3.4.2	Chiral Alcohols from Nonaromatic Ketones.....	539
20.3.4.3	Chiral Alcohols from Aromatic Ketones.....	541
20.4	Conclusion	542
	Acknowledgments	542
	References	542

20.1 INTRODUCTION

The global market of the chemical industry amounts to US \$1.8 trillion, and the contribution to this of biotechnology has been increasing year by year. According to McKinsey & Company's

report, white biotechnology accounted for only 5% of the market in 2000, but they estimate that it will increase to 10 to 20% by 2010. Biotechnology will be a significant key innovation driver in the next 10 years. As for the synthesis of various chemical compounds, biosynthesis has some advantages over conventional chemical synthesis. Reactions catalyzed by biocatalysts proceed under modest conditions with remarkable rate acceleration (up to 10^{18}), and thus applying them to industrial syntheses prevents the wasting of fossil fuels required for preparing high-temperature and high-pressure conditions that are commonly required for conventional chemical syntheses. Above all, the excellent stereoselectivities of biocatalysts enabling the formation of enantiomerically pure products are a particularly attractive feature, because the chirality of a molecule is a very important factor in the pharmaceutical field. In fact, over half of the top 100 pharmaceuticals are chiral molecules, and this chiral drug market netted over US \$145 billion in 2003. The industrial production methods for optically active compounds, regardless of the catalysts, have been reviewed by Breuer et al. [1]. There are two major strategies for the syntheses of optically active compounds: optical resolution of racemic mixtures and stereoselective conversion of prochiral structures. In this chapter, we describe recent successful examples of industrialized enzymatic chiral compound syntheses involving these two strategies.

20.2 A NOVEL TOOL FOR ENZYMATIC OPTICAL RESOLUTION—LACTONASE

20.2.1 WHAT IS A LACTONASE?

Carboxyl ester-hydrolyzing enzymes (EC 3.1.1.x) such as lipases and esterases exhibit great diversity and are the most widely used group of enzymes for industrial organic syntheses [2–4]. Reactions catalyzed by them are often stereo- and/or regioselective. In addition to hydrolysis, they catalyze several other reactions, esterification (reverse reaction of hydrolysis), and transesterification (alcoholysis of ester bonds). Some of them even exhibit catalytic activity in organic solvents, allowing the use of water-insoluble compounds as substrates [5,6]. These features make carboxyl ester hydrolases convenient to use as synthetic catalysts.

Lactonases, catalyzing hydrolysis of the intramolecular ester bonds of lactones, also belong to the esterase family. Lactones are widely found in nature as biologically active compounds and metabolic intermediates. Lactonases comprise some of the enzymes involved in the synthesis and degradation of these compounds. For example, a lactonase from *Bacillus* sp. hydrolyzes and inactivates *N*-acyl-homoserine lactones, which are produced by a range of gram-negative bacterial species as quorum-sensing signals and are involved in the cell density-dependent regulation of specific sets of genes [7–9]. Aldonate lactone hydrolases have been suggested to participate in the metabolism of aldoses [10–13]. Thus, a number of lactonases have already been found in various organisms. The reactions catalyzed by lactonases, as well as those by lipases and other esterases, are sometimes regio- and/or stereospecific, and should be applicable to the chemoenzymatic synthesis of optically active lactones and the corresponding hydroxyacids. This section describes the development of a lactonase-mediated production system. Some other potential lactonases are also dealt with.

20.2.2 OPTICAL RESOLUTION OF DL-PANTOYL LACTONE BY A FUNGAL LACTONASE

20.2.2.1 Discovery of a Novel Lactonase

D-Pantoyl lactone (D-PL) is a chiral building block for the production of calcium D-pantothenate and its derivatives, which are sold as vitamin supplements, feed additives, and cosmetics. The commercial production of D-PL relies on a chemical synthesis process that includes a complicated resolution process for DL-PL. In order to simplify the resolution

process, Shimizu et al. evaluated many microbial enzymes as potential catalysts [14–17]. At the beginning, they focused on the asymmetric reduction of a prochiral compound, ketopantoyl lactone, to yield D-PL, and screened a number of microbes for reductase activity. During the screening, they serendipitously found that some microorganisms degrade the product and accumulate D-pantoic acid. The degrading reactions, i.e., the hydrolysis of lactone, catalyzed by these microorganisms proceeded quite effectively and sometimes stereoselectively. This finding prompted them to perform intensive exploration of a novel industrial enzyme, lactonase.

PL-hydrolyzing activity is widely distributed in various microorganisms [18,19]. While bacterial strains tend to hydrolyze the L-enantiomer of PL, fungal strains such as *Fusarium*, *Gibberella*, *Penicillium*, and *Schizophyllum* preferentially hydrolyze the D-enantiomer. When an L-specific lactonase is used for the resolution of racemic PL, the optical purity of the remaining D-PL might be low, except when the hydrolysis of L-PL is completed. In the case of D-specific lactonase, D-pantoic acid of high optical purity can be constantly obtained regardless of the hydrolysis yield. After the asymmetric hydrolysis of DL-PL, the remaining L-enantiomer and D-pantoic acid formed can be separately extracted by altering the pH of the reaction mixture. The recovered L-PL is easily racemized by heating under acidic conditions and thus can be reused as a substrate (Figure 20.1).

Finally, a filamentous fungus, *Fusarium oxysporum* AKU3702, which showed the highest hydrolysis activity and stereoselectivity toward D-PL, was selected as a potential enzyme source.

20.2.2.2 Properties of the Lactonase from *Fusarium Oxysporum*

The D-PL hydrolyzing lactonase of *F. oxysporum* has been isolated and characterized in some detail [20]. The relative molecular mass of the lactonase is 125 kDa and the subunit molecular mass is 60 kDa, suggesting that the enzyme is a dimer of identical subunits. One molecule of calcium and 15.4% (w/w) glucose equivalent of carbohydrate are included in the enzyme. Calcium is necessary for both the enzyme activity and stability. The enzyme hydrolyzes aldinate lactones, such as D-galactono- γ -lactone, L-mannono- γ -lactone, and D-gulono- γ -lactone, stereospecifically. The corresponding enantiomers not only is inert as substrates but also competitively inhibit the enzyme activity. For every substrate, the reverse reaction, i.e., the lactonization of aldonic acids and D-pantoic acid, takes place under acidic pH conditions.

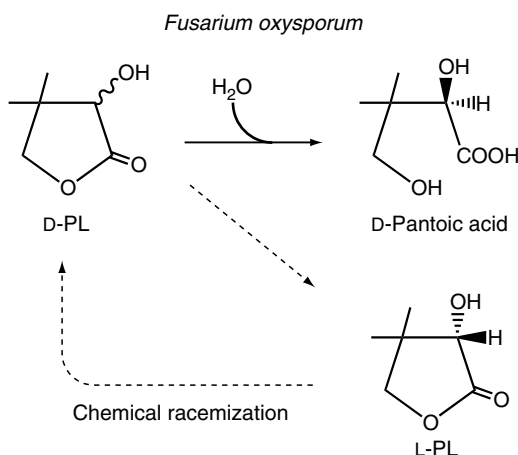


FIGURE 20.1 Principle of the optical resolution of DL-PL using the lactonase-producing fungus, *Fusarium oxysporum*.

20.2.2.3 Development of a Practical Resolution System

Under the optimum conditions, the asymmetric hydrolysis of DL-PL proceeded quite effectively with wet mycelia of *F. oxysporum* as a catalyst [18,21]. Both the concentration (>300 g/L) and the optical purity (>96% ee) of the product were sufficient for practical use. However, when the mycelia were recovered after the reaction and reused for further reactions, the residual enzyme activity was <80% of the initial level. Such a decrease in enzyme activity is a major disadvantage for a commercial process that requires retention of higher activity for long-term repeated reactions. In order to improve the enzyme stability, the *Fusarium* mycelia were immobilized by calcium alginate gel [22]. Immobilization could protect the mycelia from the damage due to stirring and the resulting leakage of the enzyme from the mycelia into the reaction mixture. It had another advantage in that the immobilized mycelia could be easily recovered after each reaction. When the calcium alginate gel-entrapped mycelia were incubated with a 300 g/L DL-PL solution with automatic control of the pH of the reaction mixture (pH 6.0 to 7.0), 90 to 95% of the D-enantiomer in the racemic mixture was selectively hydrolyzed to D-pantoic acid of high optical purity (90 to 97% ee). The immobilized mycelia retained 70% of the initial lactonase activity, even after 180 reactions (Figure 20.2; total reaction time = 3780 h). The estimated half-life of the lactonase activity of the immobilized mycelia was 6000 h, which is 35 times higher than that of the free mycelia. The improvement of the enzyme stability is partly due to calcium, as a stabilizer of the enzyme, contained in the gel.

This enzymatic resolution process was scaled up and has been in commercial operation since 1999. This process is highly satisfactory not only from economic aspects but also from environmental ones (water –49%, CO₂ –30%, and BOD –60%, compared with the former chemical resolution process). Nowadays, about 30% of the world production of calcium D-pantothenate (~6000 t/y) occurs through this chemoenzymatic process.

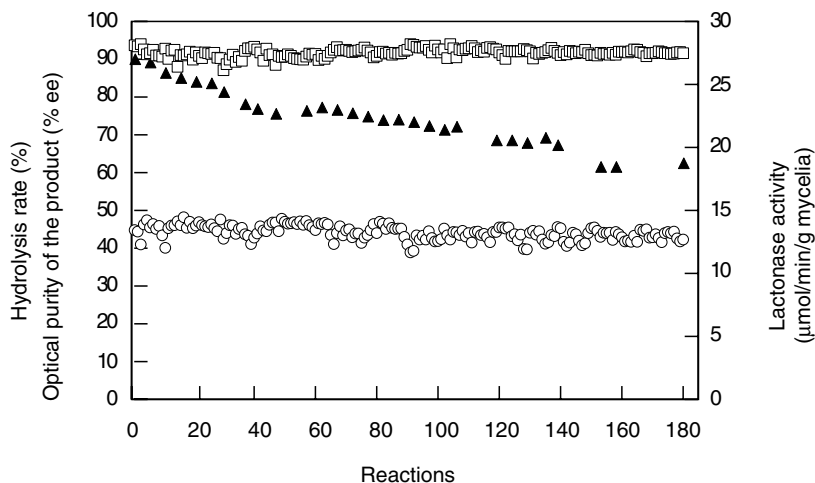


FIGURE 20.2 Asymmetric hydrolysis of DL-PL by immobilized *Fusarium oxysporum*. Batchwise reaction was repeated 180 times. Each reaction was performed as follows. Immobilized *F. oxysporum* (90 g, containing 4.6 g wet mycelia) was incubated in 100 mL of a 30% (w/v) DL-PL solution supplemented with 90 mM CaCl₂ at 30°C for 21 h. The pH of the mixture was automatically kept in the range of 6.5 to 7.0. After the reaction, immobilized cells were collected by filtration and used for the next reaction without being washed. Hydrolysis rate for DL-PL (○), optical purity of D-pantoic acid formed (□), and lactonase activity of the immobilized cells (▲). PL, pantoyl lactone.

20.2.2.4 Recent Progress

Recently, the *Fusarium* lactonase gene was overexpressed in a heterologous fungus, *Aspergillus oryzae*, under the control of an artificially modified promoter [23]. The lactonase gene includes five introns and a presumed endoplasmic reticulum (ER)-transporting signal peptide in its NH₂-terminal region. *A. oryzae* can recognize the signal peptide of the *Fusarium* lactonase and the splice junctions in the gene. Posttranslational modifications, such as transportation to the ER, cleavage of the signal peptide, and glycosylation, occurred in the same manner as in the case of *F. oxysporum*, and the recombinant enzyme was produced as a mature protein like the wild-type enzyme.

When mycelia of the recombinant *A. oryzae* were used as a catalyst for asymmetric hydrolysis of DL-PL, the initial velocity of the reaction was 30 times higher than that with *F. oxysporum*, suggesting that the transformant is promising for industrial application.

20.2.3 OTHER MICROBIAL LACTONASES

20.2.3.1 L-Pantoyl Lactone Hydrolase from *Agrobacterium Tumefaciens*

As described above, L-PL hydrolyzing activity (Figure 20.3a) was largely found in bacterial strains. Two similar but distinct L-PL hydrolases have been isolated from *Agrobacterium tumefaciens* AKU316 and *A. tumefaciens* Lu681 by Kataoka et al. [19] and Kessler et al. [24], respectively. Kessler et al. also demonstrated the availability of L-PL hydrolase for an alternative racemic PL-resolution process. The asymmetric hydrolysis of DL-PL [30% (w/v)] by the Lu681 enzyme gave D-PL, the yield being 50 to 53% and the optical purity 90 to 95% ee. Covalent immobilization in a carrier material, EupergitC, made the enzyme more stable and easier to handle in repeated batch reactions. Furthermore, mutant enzymes with improved L-PL hydrolyzing activity were generated through directed evolution. The specific activities of mutants F62S, K197D, and F100L were 2.3, 1.7, and 1.5 times higher than that of the wild-type enzyme, respectively.

20.2.3.2 Dihydrocoumarin Hydrolase from *Acinetobacter Calcoaceticus*

The dihydrocoumarin hydrolase from *Acinetobacter calcoaceticus* is a lactonase specific for aromatic lactones such as dihydrocoumarin, homogentistic acid- γ -lactone, and 2-coumaranone (Figure 20.3b) [25]. Interestingly, this enzyme is bifunctional, being capable of both the hydrolysis of ester bonds and the hydrolytic degradation of peroxoacids [26].

This enzyme also catalyzes the stereo- and/or regiospecific hydrolysis of linear esters and thus is applicable to industrial processes (Figure 20.3b) [27]. Dihydrocoumarin hydrolase catalyzes the enantiospecific hydrolysis of methyl β -acetylthioisobutyrate. The reaction product, D- β -acetylthioisobutyrate, is an important chiral building block for the synthesis of a series of angiotensin-converting enzyme inhibitors. The same enzyme is also useful for regioselective deblocking (specific for the terminal ester bond) of methyl cetraxate to yield cetraxate, which is widely used as an antiulcer agent.

20.2.3.3 (R)- δ -Decanolactone Hydrolase from *Pseudomonas* sp. 3-1

Recently, a lactonase catalyzing the enantiospecific hydrolysis of (R)- δ -decanolactone ((R)- δ -decanolactone hydrolase (DLHase), Figure 20.3c) was found in the membrane fraction of *Pseudomonas* sp. 3-1, which was isolated as a δ -decanolactone-assimilating bacterium (Honda et al., unpublished data). This enzyme was the first example of the membrane-associated lactonase. δ -Decanolactone is known as a naturally occurring aroma in meat and

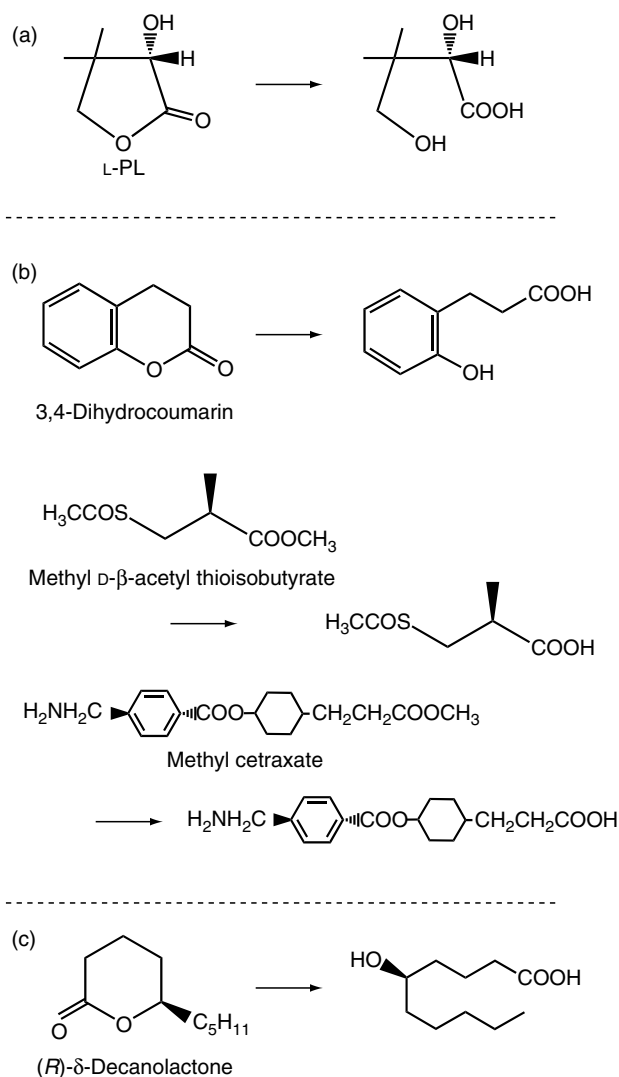


FIGURE 20.3 Hydrolysis reactions catalyzed by (a) L-pantoyl lactone hydrolase of *Agrobacterium tumefaciens*, (b) dihydrocoumarin hydrolase of *Acinetobacter calcoaceticus*, and (c) (*R*)- δ -decanolactone hydrolase of *Pseudomonas* sp. 3-1.

dairy products, and has been in great commercial demand as a food additive [28]. Besides δ -decanolactone, the enzyme hydrolyzes lactones with both 5- and 6-membered rings, such as γ -caprolactone, γ -heptalactone, γ -decanolactone, δ -valerolactone, and δ -octanolactone. The deduced amino acid sequence of DLHase exhibits low but significant similarity to those of the *Fusarium* lactonase and the δ -valerolactone hydrolase from *Comamonas* sp. NCIMB9872 [29,30]. Although they all act on intramolecular ester bonds, their substrate specificities and physiological roles are quite different from each other. This suggests that a lactonase progenitor diverged into these specific lactonases with some parallel functions independently in different evolutionary lines. Until this finding, lactonases had been classified into the same family according to their phenotype alone, i.e., catalysis of the hydrolysis of lactones, and no structural relationship had been shown among them. Structural information on these

homologous lactonases might enable the use of novel strategies, such as metagenome screening with specific probes and gene-shuffling among family proteins, to obtain potential lactonases.

20.3 DIRECT SYNTHESIS OF CHIRAL ALCOHOLS WITH STEREOSELECTIVE CARBONYL REDUCTASES

20.3.1 METHODOLOGICAL ADVANTAGES OF DIRECT SYNTHESIS

Although the optical resolution of a racemic substrate is a versatile strategy for obtaining enantiomerically pure compounds, one major disadvantage of this strategy is that the theoretical maximum yield is only 50%, because only one enantiomer in the racemic mixture can serve as the substrate.

Direct synthesis is a simple and promising method. A high efficiency, i.e., a 100% theoretical yield, can be expected. Asymmetric reduction of prochiral carbonyl substrates is one of the most competitive methodologies for chiral alcohol production. Chiral alcohols are useful starting materials as building blocks for many compounds. Among 205 patents in the area of biotransformations issued after the year 2000 by companies producing pharmaceuticals, fine chemicals, and flavours and fragrances, 57 are for alcohols, 35 of which are for technologies concerning ketone reduction [31]. This section reviews successful examples of chiral alcohol production utilizing enantioselective carbonyl reductases.

20.3.2 DIVERSITY OF CARBONYL REDUCTASES

Carbonyl compounds, such as ketones or aldehydes, are ubiquitous compounds in cells, and are important intermediates in metabolic and synthetic pathways. However, the reactivity of the carbonyl groups can cause damage to cells. The enzymes that reduce these carbonyl compounds belong to one of three enzyme superfamilies: the short-chain dehydrogenase/reductase superfamily, the medium-chain dehydrogenase/reductase superfamily, and the aldo-keto reductase superfamily. Each enzyme can be classified into one of these superfamilies based on DNA/amino acid sequences, structures, catalytic mechanisms, and so on [32–35].

As for the applicability of carbonyl reductases to chemical syntheses, it has long been well known that baker's yeast (*Saccharomyces cerevisiae*) catalyzes the asymmetric reduction of prochiral carbonyl substrates in the presence of glucose (energy source). *S. cerevisiae* cells exhibit a broad substrate specificity toward various carbonyl compounds due to the existence of many oxidoreductases. Kaluzna et al. systematically investigated 18 key reductases from baker's yeast to a total 11 α - and β -keto ester substrates [36]. Such diversity in carbonyl-reducing enzymes is convenient for cells to survive under diverse environmental conditions, but is inconvenient for practical use of the cells for chiral alcohol production. For example, the actions of more than one enzyme on a prochiral carbonyl substrate often result in a low enantiopurity of the product. Purified enzymes and heterologous microorganism cells, in which a desired reductase gene is expressed, are usually used for industrial processes.

20.3.3 COFACTOR REGENERATING SYSTEMS

20.3.3.1 Single Enzyme Systems

The reduction of carbonyl groups requires a cofactor, NAD(P)H, and a continuous supply is necessary for an efficient reaction and high conversion rate. Considering their high cost, it is not economically feasible to supply them externally in stoichiometric amounts, and thus it is desirable to construct an *in situ* regeneration system involving a second redox reaction.

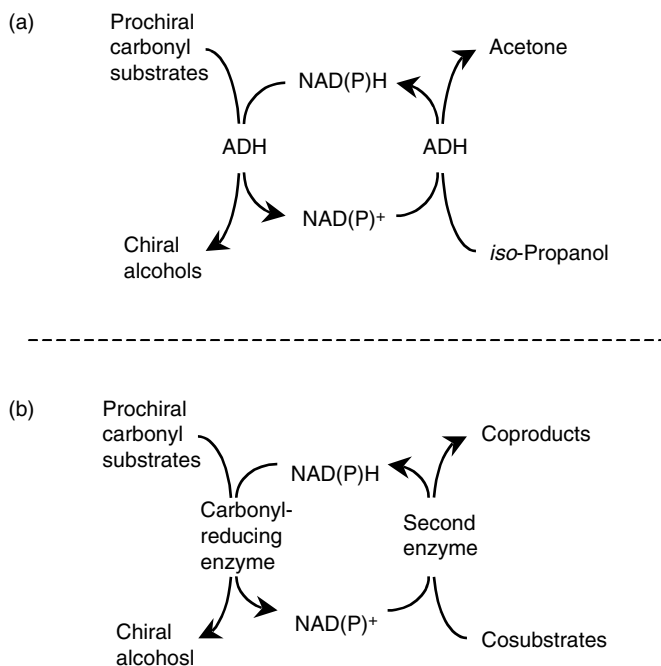


FIGURE 20.4 Cofactor regeneration systems. (a) Single enzyme (coupled substrate) system involving ADH and *iso*-propanol. ADH, alcohol dehydrogenase. (b) Coupled enzyme system.

The combination of alcohol dehydrogenase (ADH) and a small aliphatic alcohol, e.g., the ADH-*iso*-propanol system, is classic and well studied (Figure 20.4a). In this system, a single enzyme (ADH) catalyzes both the asymmetric reduction of prochiral carbonyl substrates and the regeneration of cofactors in the presence of a second substrate (*iso*-propanol). To drive the reaction equilibrium in the desired direction, a large supply of *iso*-propanol is essential. Although acetone is a problem for the thermodynamics and kinetics of the reaction, it can be easily removed from the reaction mixture. This means that the equilibrium limitations of cosubstrates can be ignored, and an increase in the conversion yield can be expected. A high concentration of *iso*-propanol increases the solubility of lipophilic substrates, but it also demands that the ADH be chemostable. Stampfer et al. reported that *Rhodococcus ruber* whole cells showed catalytic activity toward various ketones with high enantioselectivity in a 50% (v/v) *iso*-propanol-containing reaction mixture [37]. Furthermore, the ADH partially purified from this strain still retained high activity in the presence of 80% (v/v) *iso*-propanol [38].

20.3.3.2 Coupled Enzyme Systems

A two-enzyme two-substrate system means that reduction of the substrate and regeneration of the cofactor are catalyzed by different enzymes (Figure 20.4b). In this case, there is no need to consider the limitations arising from thermodynamic equilibrium described above. Furthermore, various carbonyl-reducing enzymes, not necessarily having ADH activity, can be used in this system. One good example of a second enzyme–substrate combination is the formate dehydrogenase (FDH)–formate system. Regarding this reaction, formate is a cheap and safe hydrogen donor, the coproduct is innocuous CO₂, and the reaction is irreversible. These features have allowed this system to be applied to many commercialized production systems, e.g., *L*-*tert*-leucine production in combination with leucine dehydrogenase for

reductive amination of 2-oxo-3,3-dimethylbutanoic acid [39]. Although one disadvantage of this system is that FDHs from methylotrophs substantially only use NAD^+ as a cofactor, Tishkov et al. reported pilot-scale production of a mutant FDH from *Pseudomonas* sp. 101 that showed high specificity for NADP^+ and NAD^+ [40].

The pairing of glucose dehydrogenase (GDH)–glucose is another example of a coupled enzyme system. Glucose is a cheaper cosubstrate than *iso*-propanol and formate. GDH from *Bacillus megaterium* can regenerate both NADH and NADPH [41], so it can be used together with any carbonyl-reducing enzymes independent of their cofactor dependencies. The addition of an alkaline solution is required for continuous reactions, because coproduct gluconolactone is spontaneously converted to gluconate, which decreases the pH of the reaction mixture.

Novel candidates for cofactor regeneration systems have been reported recently. Hydrogenase I from *Pyrococcus furiosus* uses molecular hydrogen as a hydrogen donor for NADPH regeneration, and forms protons as the only coproduct [42]. The enzyme retains its activity at as high as 80°C because it is from a hyperthermophilic archaeon, but the instability of NADPH becomes a limitation of reactions at such high temperature conditions.

Nakamura and Yamanaka reported a unique NADPH regeneration system involving cyanobacteria that uses light energy as reducing power [43]. Cyanobacteria are characteristic phototrophs and microbes, so they possess the features of both direct utilization of light energy and a high growth rate. This is a promising system since light energy is ultimately an economical resource.

20.3.4 PRACTICAL APPLICATIONS OF CARBONYL REDUCTASES

20.3.4.1 Production of 4-Chloro-3-Hydroxybutanoate Ethyl Ester through Alternative Processes

(*R*)- and (*S*)-4-chloro-3-hydroxybutanoate ethylesters (CHBEs) are useful chiral building blocks applicable to the synthesis of pharmaceuticals: the (*R*)-enantiomer for a precursor of L-carnitine [44] and the (*S*)-enantiomer for hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors [45]. Both enantiomers can be synthesized through asymmetric reduction of 4-chloro-3-oxobutanoate ethyl ester (COBE) (Figure 20.5). Many microorganisms and enzymes are known to possess the ability to catalyze such reduction [46–48], and some successful examples of large-scale production are listed in Table 20.1. Among them, *Candida magnoliae* showed multiple COBE-reducing enzyme activities in a cell-free extract. Three of the enzymes were purified, characterized, and named S1, S4, and R, respectively. These enzymes showed different specific activities (13.5, 7.4, and 12.2 U/mg, respectively), K_m (4.6, 0.11, and 2.9 mM), and ee [100% for (*S*), 51.1% for (*S*), and 100% for (*R*)] toward COBE [49,50]. Recombinant *Escherichia coli* coexpressing the S1- and GDH-coding genes showed (*S*)-CHBE productivity of 208 g/L in 13 h in the presence of glucose and a catalytic amount of NADP^+ [51–53]. In this mono-aqueous phase reaction, the total turnover number of NADP^+ was as high as 21,600 mol/mol. The substrate, COBE, was continuously fed at the rate of 0.02 g/min because of its instability in an aqueous environment. When an *n*-butyl acetate/water diphasic system is applied, the one-shot supply of a large amount of COBE

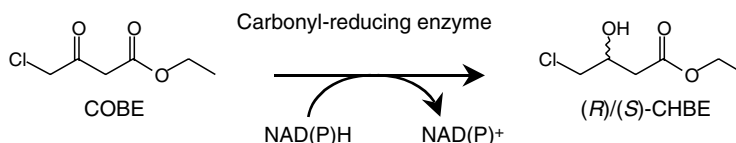


FIGURE 20.5 CHBE synthesis by asymmetric reduction of COBE. COBE, 4-chloro-3-oxobutanoate ethyl ester; CHBE, 4-chloro-3-hydroxybutanoate ethyl ester.

TABLE 20.1
Comparison of Various Systems for CHBE Production

Product	Substrate	Productivity	ee (%)	Yield (%)	Reaction System	Enzyme Source	Catalyst	Cofactor	Cofactor Regeneration	Reference
(<i>S</i>)-CHBE	COBE	100 g/L/d	>99	90.0	Aqueous	<i>Lactobacillus brevis</i>	Isolated enzyme	NADPH	<i>iso</i> -Propanol	[60]
(<i>S</i>)-CHBE	COBE	208 g/L	100	96.0	Aqueous	<i>Candida magnoliae</i>	Recombinant	NADPH	GDH-Glucose	[53]
(<i>S</i>)-CHBE	COBE	430 g/L	100	85.0	<i>n</i> -Butyl acetate/water	<i>C. magnoliae</i>	Recombinant <i>E. coli</i>	NADPH	GDH-Glucose	[53]
(<i>S</i>)-CHBE	COBE	48.7 g/L	>99	99.8	Aqueous	<i>Ralstonia eutropha</i>	Recombinant <i>E. coli</i>	NADPH	GDH-Glucose	[68]
(<i>S</i>)-CHBE	COBE	45.6 g/L	>99	91.2	Aqueous	<i>Kluyveromyces aestuarii</i>	Recombinant <i>E. coli</i>	NADH	GDH-Glucose	[69]
(<i>S</i>)-CHBE	COBE	32.2 g/L	>99	98.5	Aqueous	<i>Pichia finlandica</i>	Recombinant <i>E. coli</i>	NADH	FDH-Formate	[55]
(<i>R</i>)-CHBE	COBE	268 g/L	92	94.0	<i>n</i> -Butyl acetate/water	<i>Sporobolomyces salmonicolor</i>	Recombinant <i>E. coli</i>	NADPH	GDH-Glucose	[54]
(<i>R</i>)-CHBE	COBE	36.6 g/L	>99	95.2	Aqueous	<i>C. parapsilosis</i>	Recombinant <i>E. coli</i>	NADH	<i>iso</i> -Propanol	[55]

CHBE, 4-chloro-3-hydroxybutanoate ethyl ester; COBE, 4-chloro-3-oxobutanoate ethyl ester; GDH, glucose dehydrogenase; FDH, formate dehydrogenase.

becomes possible. In this case, 430 g/L of CHBE was produced from 500 g/L COBE in 34 h with a total turnover number of NADP^+ of 15,400 mol/mol [53]. The other enantiomer, (*R*)-CHBE, can be produced as well if the carbonyl-reducing enzyme module is substituted with that from *Sporobolomyces salmonicolor* [54].

COBE is synthesized from acetic acid by diketene, from which 5-chloro-2-pentanone (CPON) can be synthesized as well. The same recombinant *E. coli* cells as listed in Table 20.1 overexpressing the ADH-coding gene from *Pichia finlandica* could asymmetrically reduce CPON to (*R*)-5-chloro-2-pentanol (CPOL) (26.1 g/L, 99% ee), and the cells overexpressing the genes of *C. parapsilosis*-derived ADH and *Mycobacterium* sp.-derived FDH could produce (*S*)-CPOL (33.8 g/L, 98% ee) (Figure 20.6) [55].

20.3.4.2 Chiral Alcohols from Nonaromatic Ketones

Table 20.2 shows other recent examples of chiral alcohols synthesized through asymmetric reduction of nonaromatic ketones. (*R*)-1,2-Propanediol (PDO) is a major commodity chemical, and there are two biosynthetic routes for it: fermentation and asymmetric reduction of acetol. *Thermoanaerobacterium thermosaccharolyticum* (previously known as *Clostridium thermosaccharolyticum*) is one of the best strains for the fermentation route. This strain can produce PDO through the fermentation of various sugars, such as D-glucose, D-xylose, D-mannose, L-arabinose, cellobiose, and galactose [56,57]. The highest productivity was obtained by Sánchez-Riera et al., i.e., a PDO titer of 9.05 g/L with a yield with glucose of 0.2 g/g, although the major product was lactate (11.1 g/L, 0.24 g/g glucose) [58].

Concerning hexanediol production, the substrate diketone is subjected to two-step asymmetric reduction. The selectivity regarding hydroxyketones produced in the first reduction step and diols in the second one is an important factor that affects downstream processing. In addition to the continuous production process for (2*R*,5*R*)-hexanediol described in Table 20.2, (2*S*,5*S*)-hexanediol production by fed batch reduction with *S. cerevisiae* has been achieved by Jülich Fine Chemicals GmbH on a 10 kg scale (conversion 95%, selectivity 68%, ee > 99%, de > 90%) [59].

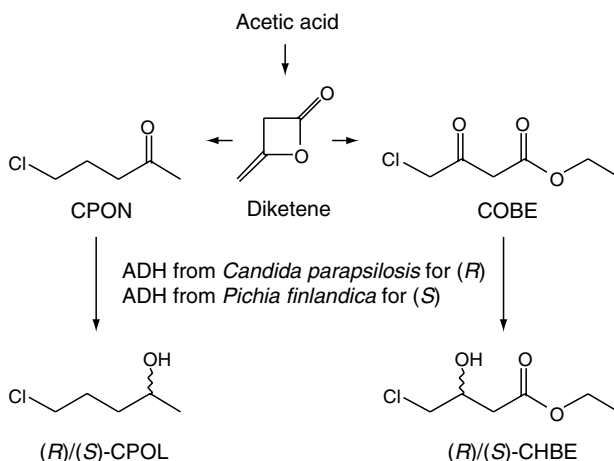


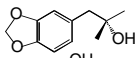
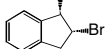
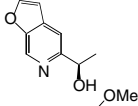
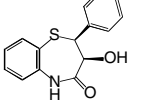
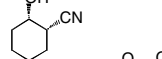
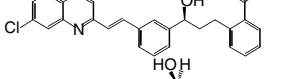
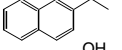
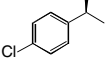

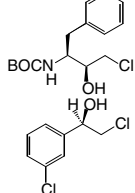
FIGURE 20.6 Production of chiral secondary alcohols with both configurations. ADH, alcohol dehydrogenase; CPON, 5-chloro-2-pentanone; CPOL, 5-chloro-2-pentanol; COBE, 4-chloro-3-oxobutanoate ethyl ester; CHBE, 4-chloro-3-hydroxybutanoate ethyl ester.

Eckstein et al. recently reported (*R*)-2-octanol production from 2-octanone using ADH from *Lactobacillus brevis* as a catalyst and *iso*-propanol as a cosubstrate (88% conversion, >99% ee) [60]. The reaction proceeded in a biphasic system comprising buffer and an ionic liquid [BMIM and (CF₃SO₂)₂N]. As the ionic liquid removed acetone from the aqueous buffer phase, the reaction equilibrium shifted to cofactor regeneration, and thus resulted in a high reaction rate.

20.3.4.3 Chiral Alcohols from Aromatic Ketones

Chiral alcohols that are synthesized from aromatic ketones are listed in Table 20.3. These compounds with complex structures are important chiral synthons for optically active drugs that are synthesized through multistep enzyme reactions (not fermentation). The time-to-market factor is often given priority over productivity in pharmaceuticals. Several good reviews have been published on the synthesis of chiral drug intermediates by means of biocatalyst-involved methods [61–63].

TABLE 20.3
Chiral Alcohols from Aromatic Ketones

Product	Productivity	ee%	Yield%	Enzyme Source	Catalyst	Reference
	75 g/L/d	>99.9	>95	<i>Zygosaccharomyces rouxii</i>	Whole cells	[78]
	2.25 g/L	99	75	<i>Saccharomyces cerevisiae</i>	Whole cells	[79]
	17.5 g/L	97	99	<i>Candida maris</i>	Whole cells	[80]
	91.5 g/L	99	89.5	<i>C. maris</i>	Cell-free extract	
	80 g/L	99	80	<i>S. cerevisiae</i>	Whole cells	[81]
	8.6 g/L/d	93	85	<i>S. montanus</i>	Whole cells	[82]
	0.5 g/L	>95	67	<i>Microbacterium</i> sp.	Whole cells	[83]
	118 g/L/d	99	71	<i>C. parapsilosis</i>	Isolated enzyme	[84]
	19.7 g/L	99	63	<i>Rhodococcus erythropolis</i>	Isolated enzyme	[85]
	20.8 g/L	99	65	<i>R. ruber</i>	Whole cells	[86]
	0.64 g/L	99.8 ee 99 de	80	<i>Streptomyces nodosus</i>	Whole cells	[87]
	130 g/L	>99	87	<i>Rhodotorula glutinis</i>	Cell-free extract	

20.4 CONCLUSION

Whereas the excellent specificities of enzymes (substrate-, stereo-, and/or regiospecificities) make them attractive catalysts for chiral syntheses, the narrow substrate tolerances of enzymes due to their strict specificities are a major disadvantage and, thus, a double-edged sword from the industrial point of view. Various novel methodologies enhanced by advanced molecular biological techniques have been proposed to overcome such limitations. Metagenome analysis in conjunction with bioinformatics and high-throughput screening is a powerful approach for exploring potential enzymes in biological niches [64,65]. Another approach is expansion of the “reaction-specificities” of known enzymes. There have been a number of reports on alteration of the catalytic properties of enzymes by means of protein engineering techniques, and on the application of “old” enzymes with or without such artificial customization to “new” processes [66,67]. These new technologies together with traditional screening should allow further applicability of enzymes to chiral syntheses.

ACKNOWLEDGMENTS

The works in our laboratory were supported in part by a Grant-in-Aid for Scientific Research (No. 15658024, to S.S.) from the Ministry of Education, Science, Sports, and Culture, Japan, the COE for Microbial-Process Development Pioneering Future Production Systems (COE program of the Ministry of Education, Science, Sports, and Culture, Japan, to S.S.), and the Project for the Development of a Technological Infrastructure for Industrial Bioprocesses on R&D of New Industrial Science and Technology Frontiers (to S.S.) of the New Energy and Industrial Technology Development Organization (NEDO), Japan. We wish to thank Daiichi Fine Chemical (Takaoka), Kaneka (Osaka), Lonza (Visp), and DSM Vitamins (Keiseraugust) for their collaboration.

REFERENCES

1. Breuer, M., Ditrich, K., Habicher, T., et al., Industrial methods for the production of optically active intermediates, *Angew. Chem. Int. Ed.*, 43(7), 788–824, 2004.
2. Bornscheuer, U.T. and Kazlauskas, R.J., Hydrolases, in *Organic Synthesis: Regio- and Stereoselective Biotransformations*, Wiley-VCH, Weinheim, Germany, 1999.
3. Bornscheuer, U.T., Microbial carboxyl esterases: classification, properties and application in biocatalysis, *FEMS Microbiol. Rev.*, 26(1), 73–81, 2002.
4. Reetz, M.T., Lipase as practical biocatalysts, *Curr. Opin. Chem. Biol.*, 6(2), 145–150, 2002.
5. Klivanov, A.M., Enzymatic catalysis in anhydrous organic solvents, *Trends Biochem. Sci.*, 14(4), 141–144, 1989.
6. Klivanov, A.M., Improving enzymes by using them in organic solvents, *Nature*, 409(6817), 241–246, 2001.
7. Dong, Y.H., Wang, L.H., Xu, J.L., et al., Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase, *Nature*, 411(6839), 813–817, 2001.
8. Dong, Y.H., Gusti, A.R., Zhang, Q., Xu, J.L., et al., Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species, *Appl. Environ. Microbiol.*, 68(4), 1754–1759, 2002.
9. Lee, S.J., Park, S.Y., Lee, J.J., et al., Genes encoding the *N*-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*, *Appl. Environ. Microbiol.*, 68(8), 3919–3924, 2002.
10. Bublitz, C. and Lehninger, A.L., The role of aldololactonase in the conversion of L-gulonate to L-ascorbate, *Biochim. Biophys. Acta*, 47(2), 288–297, 1961.
11. Dilworth, M.J., Arwas, R., McKay, I.A., et al., Pentose metabolism in *Rhizobium leguminosarum* MNF300 and in cowpea *Rhizobium* NGR234, *J. Gen. Microbiol.*, 132(10), 2733–2742, 1986.

12. Novick, N.J. and Tyler, M.E., L-Arabinose metabolism in *Azospirillum brasiliense*, *J. Bacteriol.*, 149(1), 364–367, 1982.
13. Rigo, L.U., Marechal, L.R., Vieira, M.M., et al., Oxidative pathway for L-rhamnose degradation in *Pullularia pullulans*, *Can. J. Microbiol.*, 31(9), 817–822, 1985.
14. Shimizu, S., Hata, H., and Yamada, H., Reduction of ketopantoyl lactone to D-(–)-pantoyl lactone by microorganisms, *Agric. Biol. Chem.*, 48(9), 2285–2291, 1984.
15. Shimizu, S., Hattori, S., Hata, H., et al., One-step microbial conversion of a racemic mixture of pantoyl lactone to optically active D-(–)-pantoyl lactone, *Appl. Environ. Microbiol.*, 53(3), 519–522, 1987.
16. Hata, H., Shimizu, S., and Yamada, H., Enzymatic production of D-(–)-pantoyl lactone from ketopantoyl lactone, *Agric. Biol. Chem.*, 51(11), 3011–3016, 1987.
17. Kataoka, M., Shimizu, S., and Yamada, H., Novel enzymatic production of D-(–)-pantoyl lactone through the stereo-specific reduction of keto pantoic acid, *Agric. Biol. Chem.*, 54(1), 177–182, 1990.
18. Kataoka, M., Shimizu, K., Sakamoto, K., et al., Lactonohydrolase-catalyzed optical resolution of pantoyl lactone: selection of a potent enzyme producer and optimization of culture and reaction conditions for practical resolution, *Appl. Microbiol. Biotechnol.*, 44(3–4), 333–338, 1995.
19. Kataoka, M., Nomura, J., and Shinohara, M., Purification and characterization of a novel lactonohydrolase from *Agrobacterium tumefaciens*, *Biosci. Biotechnol. Biochem.*, 64(6), 1255–1262, 2000.
20. Shimizu, S., Kataoka, M., Shimizu, K., et al., Purification and characterization of a novel lactonohydrolase, catalyzing the hydrolysis of aldinate lactones and aromatic lactones, from *Fusarium oxysporum*, *Eur. J. Biochem.*, 209(1), 383–390, 1992.
21. Kataoka, M., Shimizu, K., Sakamoto, K., et al., Optical resolution of racemic pantolactone with a novel fungal enzyme, lactonohydrolase, *Appl. Microbiol. Biotechnol.*, 43(6), 974–977, 1995.
22. Sakamoto, K., Honda, K., Wada, K., et al., Practical resolution system for DL-pantoyl lactone using the lactonase from *Fusarium oxysporum*, *J. Biotechnol.*, 118, 99–106, 2005.
23. Honda, K., Tsuboi, H., Minetoki, T., et al., Expression of the *Fusarium oxysporum* lactonase gene in *Aspergillus oryzae*—molecular properties of the recombinant enzyme and its application, *Appl. Microbiol. Biotechnol.*, 66(5), 520–526, 2005.
24. Kessler, M., Friedrich, T., Höffken, H.W., et al., Development of a novel biocatalyst for the resolution of *rac*-pantolactone, *Adv. Synth. Catal.*, 344(10), 1103–1110, 2002.
25. Kataoka, M., Honda, K., and Shimizu, S., 3,4-Dihydrocoumarin hydrolase with haloperoxidase activity from *Acinetobacter calcoaceticus*, *Eur. J. Biochem.*, 267(1), 3–10, 2000.
26. Honda, K., Kataoka, M., Sakuradani, E., et al., Role of *Acinetobacter calcoaceticus* 3,4-dihydrocoumarin hydrolase in oxidative stress defence against peroxoacids, *Eur. J. Biochem.*, 270(3), 486–494, 2003.
27. Honda, K., Kataoka, M., and Shimizu, S., Enzymatic preparation of D-β-acetylthioisobutyric acid and cetaxate hydrochloride using a stereo- and/or regioselective hydrolase, 3,4-dihydrocoumarin hydrolase from *Acinetobacter calcoaceticus*, *Appl. Microbiol. Biotechnol.*, 60(3), 288–292, 2002.
28. Adams, T.B., Greer, D.B., Doull, J., et al., The FEMA GRAS assessment of lactones used as flavour ingredients, *Food Chem. Toxicol.*, 36(4), 249–278, 1998.
29. Onakunle, O.A., Knowles, C.J., and Bunch, A.W., The formation and substrate specificity of bacterial lactonases capable of enantioselective resolution of racemic lactones, *Enzyme Microb. Technol.*, 21(4), 245–251, 1997.
30. Iwaki, H., Hasegawa, Y., Wang, S., et al., Cloning and characterization of a gene cluster involved in cyclopentanol metabolism in *Comamonas* sp. strain NCIMB 9872 and biotransformations effected by *Escherichia coli*-expressed cyclopentanone 1,2-monooxygenase, *Appl. Environ. Microbiol.*, 68(11), 5671–5684, 2002.
31. Panke, S., Held, M., and Wubbolts, M., Trends and innovations in industrial biocatalysis for the production of fine chemicals, *Curr. Opin. Biotechnol.*, 15(4), 272–279, 2004.
32. Jörnvall, H., Höög, J.-O., and Persson, B., SDR and MDR: completed genome sequences show these protein families to be large, of old origin, and of complex nature, *FEBS Lett.*, 445(2–3), 261–264, 1999.
33. Forrest, G.L. and Gonzalez, B., Carbonyl reductase, *Chem. Biol. Interact.*, 129(1–2), 21–40, 2000.
34. Ellis, E.M., Microbial aldo-keto reductases, *FEMS Microbiol. Lett.*, 216(2), 123–131, 2002.
35. Hyndman, D., Bauman, D.R., Heredia, V.V., et al., The aldo-keto reductase superfamily home-page, *Chem. Biol. Interact.*, 143–144, 621–631, 2003.

36. Kaluzna, I.A., Matsuda, T., Sewell, A.K., et al., Systematic investigation of *Saccharomyces cerevisiae* enzymes catalyzing carbonyl reductions, *J. Am. Chem. Soc.*, 126(40), 12827–12832, 2004.
37. Stampfer, W., Kosjek, B., Moitzi, C., et al., Biocatalytic asymmetric hydrogen transfer, *Angew. Chem. Int. Ed.*, 41(6), 1014–1017, 2002.
38. Kroutil, W., Mang, H., Edegger, K., et al., Recent advances in the biocatalytic reduction of ketones and oxidation of *sec*-alcohols, *Curr. Opin. Chem. Biol.*, 8(2), 120–126, 2004.
39. Kula, M.-R. and Kragl, U., Dehydrogenases in the synthesis of chiral compounds, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, pp. 839–866.
40. Tishkov, V.I., Galkin, A.G., Fedorchuk, V.V., et al., Pilot scale production and isolation of recombinant NAD⁺- and NADP⁺-specific formate dehydrogenases, *Biotechnol. Bioeng.*, 64(2), 187–193, 1999.
41. Makino, Y., Ding, J.-Y., Negoro, S., et al., Purification and characterization of a new glucose dehydrogenase from vegetative cells of *Bacillus megaterium*, *J. Ferment. Bioeng.*, 67(6), 374–379, 1989.
42. Mertens, R., Greiner, L., van den Banb, E.C.D., et al., Practical applications of hydrogenase I from *Pyrococcus furiosus* for NADPH generation and regeneration, *J. Mol. Catal. B Enzym.*, 24–25, 39–52, 2003.
43. Nakamura, K. and Yamanaka, R., Light mediated cofactor recycling system in biocatalytic asymmetric reduction of ketone, *Chem. Commun.*, (16), 1782–1783, 2002.
44. Zhou, B., Gopalan, A.S., VanMiddlesworth, F., et al., Stereochemical control of yeast reductions. 1. Asymmetric synthesis of L-carnitine, *J. Am. Chem. Soc.*, 105(18), 5925–5926, 1983.
45. Karanewsky, D.S., Badia, M.C., Ciosek, C.P.J., et al., Phosphorus-containing inhibitors of HMG-CoA reductase. 1. 4-[2-arylethyl]-hydroxyphosphinyl-3-hydroxybutanoic acids: a new class of cell-selective inhibitors of cholesterol biosynthesis, *J. Med. Chem.*, 33(10), 2925–2956, 1990.
46. Yasohara, Y., Kizaki, N., Hasegawa, J., et al., Synthesis of optically active ethyl 4-chloro-3-hydroxybutanoate by microbial reduction, *Appl. Microbiol. Biotechnol.*, 51(6), 847–851, 1999.
47. Shimizu, S., Kataoka, M., and Kita, K., Chiral alcohol synthesis with microbial carbonyl reductases in a water-organic solvent two-phase system, *Ann. NY Acad. Sci.*, 864(1), 87–95, 1998.
48. Kataoka, M., Kita, K., Wada, M., et al., Novel bioreduction system for the production of chiral alcohols, *Appl. Microbiol. Biotechnol.*, 62(5–6), 437–445, 2003.
49. Wada, M., Kawabata, H., Kataoka, M., et al., Purification and characterization of an aldehyde reductase from *Candida magnoliae*, *J. Mol. Catal. B Enzym.*, 6(3), 333–339, 1999.
50. Wada, M., Kataoka, M., Kawabata, H., et al., Purification and characterization of NADPH-dependent carbonyl reductase, involved in stereoselective reduction of ethyl 4-chloro-3-oxobutanoate, from *Candida magnoliae*, *Biosci. Biotechnol. Biochem.*, 62(2), 280–285, 1998.
51. Yasohara, Y., Kizaki, N., Hasegawa, J., et al., Molecular cloning and overexpression of the gene encoding an NADPH-dependent carbonyl reductase from *Candida magnoliae*, involved in stereoselective reduction of ethyl 4-chloro-3-oxobutanoate, *Biosci. Biotechnol. Biochem.*, 64(7), 1430–1436, 2000.
52. Yasohara, Y., Kizaki, N., Hasegawa, J., et al., Stereoselective reduction of alkyl 3-oxobutanoate by carbonyl reductase from *Candida magnoliae*, *Tetrahedron Asymmetry*, 12(12), 1713–1718, 2001.
53. Kizaki, N., Yasohara, Y., Hasegawa, J., et al., Synthesis of optically pure ethyl (S)-4-chloro-3-hydroxybutanoate by *Escherichia coli* transformant cells coexpressing the carbonyl reductase and glucose dehydrogenase genes, *Appl. Microbiol. Biotechnol.*, 55(5), 590–595, 2001.
54. Kataoka, M., Yamamoto, K., Kawabata, H., et al., Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by *Escherichia coli* transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes, *Appl. Microbiol. Biotechnol.*, 51(4), 486–490, 1999.
55. Matsuyama, A., Yamamoto, H., and Kobayashi, Y., Practical application of recombinant whole-cell biocatalysts for the manufacturing of pharmaceutical intermediates such as chiral alcohols, *Org. Process Res. Dev.*, 6(4), 558–561, 2002.
56. Cameron, D.C., Altaras, N.E., Hoffman, M.L., et al., Metabolic engineering of propanediol pathways, *Biotechnol. Prog.*, 14(1), 116–125, 1998.
57. Cameron, D.C. and Cooney, C.L., A novel fermentation: the production of R(–)-1,2-propanediol and acetol by *Clostridium thermosaccharolyticum*, *Bio/Technology*, 4, 651–654, 1986.
58. Sánchez-Riera, F., Cameron, D.C., and Cooney, C.L., Influence of environmental factors in the production of R(–)-1,2-propanediol by *Clostridium thermosaccharolyticum*, *Biotechnol. Lett.*, 9(7), 449–454, 1987.

59. <http://www.juelich-chemicals.de/>
60. Eckstein, M., Filho, M.V., Liesec, A., et al., Use of an ionic liquid in a two-phase system to improve an alcohol dehydrogenase catalysed reduction, *Chem. Commun.*, (9), 1084–1085, 2004.
61. Patel, R.N., Microbial/enzymatic synthesis of chiral drug intermediates, *Adv. Appl. Microbiol.*, 47, 33–78, 2000.
62. Node, M., Development of new methods in asymmetric reactions and their applications, *Yakugaku Zasshi*, 122(1), 71–88, 2002.
63. Patel, R.N., Microbial/enzymatic synthesis of chiral pharmaceutical intermediates, *Curr. Opin. Drug Dis. Devel.*, 6(6), 902–920, 2003.
64. Lorenz, P., Lieveton, K., Niehaus, F., et al., Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space, *Curr. Opin. Biotechnol.*, 13(6), 572–577, 2002.
65. Streit, W.R. and Schmitz, R.A., Metagenomics — the key to the uncultured microbes, *Curr. Opin. Microbiol.*, 7(5), 492–498, 2004.
66. Bornscheuer, U.T. and Kazlauskas, R.J., Catalytic promiscuity in biocatalysis: using old enzymes to form new bonds and follow new pathway, *Angew. Chem. Int. Ed.*, 43(45), 6032–6040, 2004.
67. Faber, K. and Kroutil, W., New enzymes for biotransformations, *Curr. Opin. Chem. Biol.*, 9, 174–180, 2005.
68. Yamamoto, H., Matsuyama, A., and Kobayashi, Y., Synthesis of ethyl (S)-4-chloro-3-hydroxybutanoate using *fabG*-homologues, *Appl. Microbiol. Biotechnol.*, 61(2), 133–139, 2003.
69. Yamamoto, H., Mitsuhashi, K., Kimoto, N., et al., A novel NADH-dependent carbonyl reductase from *Kluyveromyces aestuarii* and comparison of NADH-regeneration system for the synthesis of ethyl (S)-4-chloro-3-hydroxybutanoate, *Biosci. Biotechnol. Biochem.*, 68(3), 638–649, 2004.
70. Wada, M., Kawabata, H., Yoshizumi, A., et al., Occurrence of multiple ethyl 4-chloro-3-oxobutanoate-reducing enzymes in *Candida magnoliae*, *J. Biosci. Bioeng.*, 87(2), 144–148, 1999.
71. Kometani, T., Yoshii, H., Takeuchi, Y., et al., Large-scale preparation of (R)-1,2-propanediol through baker's yeast-mediated bioreduction, *J. Ferment. Bioeng.*, 76(5), 414–415, 1993.
72. Yamada-Onodera, K., Kawahara, N., Tani, Y., et al., Synthesis of optically active diols by *Escherichia coli* transformant cells that express the glycerol dehydrogenase gene of *Hansenula polymorpha* DL-1, *Eng. Life Sci.*, 4(5), 413–417, 2004.
73. Haberland, J., Hummel, W., Daussmann, T., et al., New continuous production process for enantiopure (2R,5R)-hexanediol, *Org. Process Res. Dev.*, 6(4), 458–462, 2002.
74. Cappaert, L. and Larroche, C., Behaviour of dehydrated baker's yeast during reduction reactions in a biphasic medium, *Appl. Microbiol. Biotechnol.*, 64(5), 686–690, 2004.
75. Kometani, T., Yoshii, H., Kitatsuji, E., et al., Large-scale preparation of (S)-ethyl 3-hydroxybutanoate with a high enantiomeric excess through baker's yeast-mediated bioreduction, *J. Ferment. Bioeng.*, 76(1), 33–37, 1993.
76. Amidjojo, M., Nowak, A., Franco-Lara, E., et al., Abstracts of papers, 2nd International Congress on Biocatalysis, Hamburg, Germany, Aug 29–Sept 1, 2004, p. 157.
77. Wolberg, M., Hummel, W., Wandrey, C., et al., Highly regio- and enantioselective reduction of 3,5-dioxocarboxylates, *Angew. Chem.*, 112(23), 4476–4478, 2000.
78. Vicenzi, J.T., Zmijewski, M.J., Reinhard, M.R., et al., Large-scale stereoselective enzymatic ketone reduction with in situ product removal via polymeric adsorbent resins, *Enzyme Microbial. Technol.*, 20(7), 494–499, 1997.
79. Aleu, J., Fronza, G., Fuganti, C., et al., On the baker's yeast mediated transformation of α -bromoones: synthesis of (1S,2R)-2-bromoindan-1-ol and (2S,3S)-3-bromo-4-phenylbutan-2-ol, *Tetrahedron Asymmetry*, 9(9), 1589–1596, 1998.
80. Kawano, S., Horikawa, M., Yasohara, Y., et al., Microbial enantioselective reduction of acetylpyridine derivatives, *Biosci. Biotechnol. Biochem.*, 67(4), 809–814, 2003.
81. Kometani, T., Sakai, Y., Matsumae, H., et al., Production of (2S,3S)-2,3-dihydro-3-hydroxy-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(W)-one, a key intermediate for diltiazem synthesis, by baker's yeast-mediated reduction, *J. Ferment. Bioeng.*, 84(3), 195–199, 1997.
82. Dehli, J.R. and Gotor, V., Dynamic kinetic resolution of 2-oxocycloalkanecarbonitriles: chemo-enzymatic syntheses of optically active cyclic β - and γ -amino alcohols, *J. Org. Chem.*, 67(19), 6816–6819, 2002.

83. Roberge, C., King, A., Pecore, V., et al., Asymmetric bioreduction of a keto ester to its corresponding (*S*)-hydroxy ester by *Microbacterium* sp. MB 5614, *J. Ferment. Bioeng.*, 81(6), 530–533, 1996.
84. Zelinski, T., Liese, A., Wandrey, C., et al., Asymmetric reductions in aqueous media: enzymatic synthesis in cyclodextrin containing buffers, *Tetrahedron Asymmetry*, 10(9), 1681–1687, 1999.
85. Groeger, H., Hummel, W., Buchholz, S., et al., Practical asymmetric enzymatic reduction through discovery of a dehydrogenase-compatible biphasic reaction media-176, *Org. Lett.*, 5(2), 173–176, 2003.
86. Stampfer, W., Edegger, K., Kosjek, B., et al., Simple biocatalytic access to enantiopure (*S*)-1-heteroarylethanol employing a microbial hydrogen transfer reaction, *Adv. Synth. Catal.*, 346, 57–62, 2004.
87. Patel, R.N., Banerjee, A., McNamee, C.G., et al., Preparation of chiral synthon for HIV protease inhibitor: stereoselective microbial reduction of *N*-protected α -aminochloroketone, *Tetrahedron Asymmetry*, 8(15), 2547–2552, 1997.
88. Kizaki, N., Sawa, I., Yano, M., et al., Purification and characterization of a yeast carbonyl reductase for synthesis of optically active (*R*)-styrene oxide derivatives, *Biosci. Biotechnol. Biochem.*, 69(1), 79–86, 2005.

21 Discovery of Arylmalonate Decarboxylase and Conversion of the Function by Rational Design

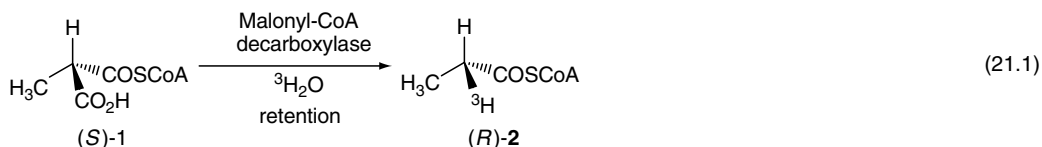
Kenji Miyamoto and Hiromichi Ohta

CONTENTS

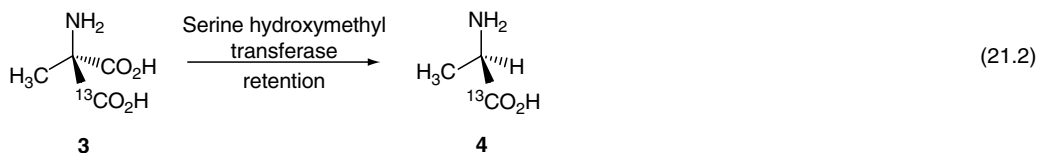
21.1	Introduction	547
21.2	Asymmetric Decarboxylation of α -Aryl- α -Methylmalonate	548
21.2.1	Screening of Microorganism	549
21.2.2	Substrate Specificity	549
21.2.3	Purification and Characterization of the Enzyme	551
21.2.4	Cloning of AMDase Gene.....	553
21.2.5	Site-Directed Mutagenesis of AMDase Gene.....	554
21.2.6	Stereochemical Course of the Reaction.....	555
21.3	Conversion of the Function by Rational Design	558
21.3.1	Inversion of Enantioselectivity of AMDase	558
	References	560

21.1 INTRODUCTION

Decarboxylation of malonic acid is a well-known process in the biosynthesis of long-chain fatty acids starting from acetic acid. If this kind of enzymes exhibits enantioselectivity for α -substituted malonates, it will be useful as a novel method for preparing optically active carboxylic acids. Indeed malonyl-CoA decarboxylase from uropygial gland is enantioselective to the substrate and the product. Racemate of methylmalonyl-CoA (**1**) was incubated with the above decarboxylase in $^3\text{H}_2\text{O}$. (*S*)-Enantiomer was smoothly decarboxylated to result in 2-(^3H)-propionyl-CoA (**2**), while (*R*)-isomer of the substrate remained intact (Equation 21.1). The absolute configuration of α -carbon of resulting **2** was revealed to be (*R*). Thus, the decarboxylase distinguishes the chirality of **1** and gives enantiomerically pure (*R*)-isomer by retention of configuration [1].



Serine hydroxymethyltransferase (SHMT) catalyzes a similar reaction. This enzyme decarboxylates α -amino- α -methylmalonate by the aid of pyridoxal-5'-phosphate (PLP), and is unusual in that it promotes a wide range of different types of reactions of α -amino acids [2]. It is capable of catalyzing aldol/retro-aldol reaction and transamination in addition to decarboxylation reaction. This is due to the similarity of the reaction pattern. The initial step of each reaction is the decomposition of the Schiff base formed between the substrate and pyridoxal coenzyme. The decarboxylation of α -amino- α -methylmalonate (**3**) catalyzed by SHMT is enantioselective. Thomas et al. synthesized chiral **3**, containing ^{13}C in one of the two carboxyl groups. Both enantiomers of **3** were incubated with SHMT in the presence of PLP. When (*R*)-isomer was used, no ^{13}C was found in the product. On the contrary, ^{13}C was retained in the resulting alanine (**4**) when (*S*)-enantiomer was employed as the starting material. Apparently, pro-(*R*) carboxyl group of α -amino- α -methylmalonate was removed as carbon dioxide. The absolute configuration of resulting alanine is (*R*), indicating that the stereochemical course of the reaction is retention of configuration, as illustrated in Equation 21.2 [2]. α -Aminomalonnate decarboxylase utilized a similar mechanism. In this case, however, the starting material is prone to racemize under reaction conditions, and careful experiments to evaluate the stereochemistry of the reaction are required [3].



21.2 ASYMMETRIC DECARBOXYLATION OF α -ARYL- α -METHYLMALONATE

Optically active α -arylpropionic acids are useful compounds as anti-inflammatory agents (Figure 21.1) [4,5]. We intended to synthesize such optically active compounds by asymmetric decarboxylation of α -aryl- α -methylmalonates with the aid of biocatalysis, and have found that *Alcaligenes bronchisepticus* was the most active strain. Unfortunately the absolute configuration of the product was opposite to that of the above-mentioned active pharmaceuticals. Thus, this reaction is considered to be of little value for obtaining such compounds. However, as this is a new type of biotransformation that can be carried out in a preparative scale and the optical and chemical yields were high, it is expected that this decarboxylation reaction would be utilized to other objectives and be a good model for examining the interaction between an enzyme and synthetic substrates. Therefore, the enzyme was isolated and studied in this direction.

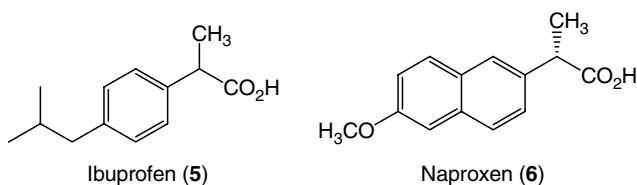


FIGURE 21.1 Nonsteroidal anti-inflammatory 2-arylpropionates.

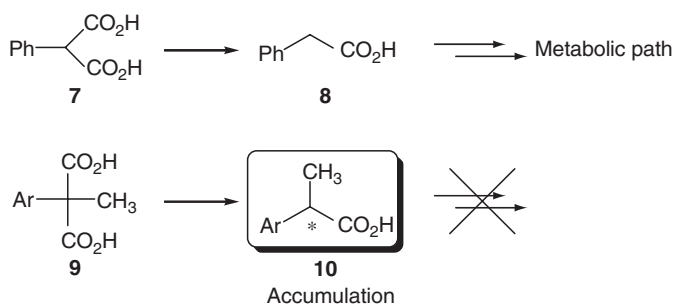


FIGURE 21.2 Screening of microorganisms.

21.2.1 SCREENING OF MICROORGANISM

Screening of a microorganism that is capable of decarboxylating α -aryl- α -methylmalonates was carried out using a medium containing phenylmalonate (7) as the sole source of carbon, because we supposed that the first step of the metabolic path of the acid would be decarboxylation to give phenylacetate (8), which would further be metabolized by oxidation of α -position (Figure 21.2). Accordingly, the microorganisms that assimilate the added carbon source and grow on this medium are expected to have decarboxylase, which is active to phenylmalonate (7). At least a few of the enzymes that catalyze decarboxylation of 7 can be expected to be active to α -aryl- α -methylmalonates (9), as the difference in the structure between two molecules is only the presence or the absence of a methyl group on the α -position. If the presence of a methyl group conveniently inhibits the following metabolic degradation, the expected monoacid would be obtained. A wide variety of soil samples and type cultures were tested and we found a few strains that were capable of growing on the medium. We selected a bacterium identified as *A. bronchisepticus* KU1201, because this strain gave optically active α -arylpropionate (10) starting from α -aryl- α -methylmalonate (9) [6,7]. This enzyme was revealed to be an inducible enzyme, the inducer being phenylmalonate (7).

21.2.2 SUBSTRATE SPECIFICITY

A medium (50 mL) containing phenylmalonate (250 mg) and peptone (50 mg), yeast extract, and some inorganic salts was inoculated with *A. bronchisepticus* and shaken for 4 d at 30°C. The substrate, 250 mg of α -methyl- α -phenylmalonate (9a), was added to the resulting suspension and the incubation was continued for an additional 5 d. The mixture was acidified, saturated with NaCl, and extracted with ether. After removal of the solvent, the organic residue was treated with an excess amount of diazomethane and the expected product was isolated as its methyl ester. Purification with preparative TLC afforded optically active methyl α -phenylpropionate. The absolute configuration was proved to be (*R*) by the specific rotation and the enantiomeric excess (ee) was determined to be 98% by HPLC using a column with an optically active solid phase (Table 21.1). The enzyme system of *A. bronchisepticus* was also effective to other compounds with a substituent on the phenyl ring. As is clear from Table 21.1, an electron-withdrawing substituent (9b) is preferable to promote the reaction. The enzyme also accepted β -naphthyl ring (9d) and thienyl ring (9e) as good substrates. No decarboxylation was observed when the malonate had a substituent on the *ortho*-position of the aryl ring (9f), or in the case when the alkyl group of α -position was ethyl instead of methyl (Figure 21.3). The fact that the compound with an α -naphthyl ring (9h) is inactive to the enzyme system is a marked contrast to the fact that 9d is a good substrate. The inactivity of all these compounds may be due to the steric effect rather than electronic effect. One possible key

TABLE 21.1
Substrate Specificity

$$\text{Ar}-\text{CH}(\text{R})-\text{CO}_2\text{H} \xrightarrow{\text{Alcaligenes bronchisepticus}} \text{Ar}-\text{CH}(\text{R})-\text{CO}_2\text{H}$$

Compound	Ar	R	Sub. conc. (%)	Yield (%)	ee (%)
9a		CH ₃	0.5	80	98
9b		CH ₃	0.5	95	98
9c		CH ₃	0.1	48	99
9d		CH ₃	0.5	96	>95
9e		CH ₃	0.3	98	95

to an appropriate interpretation is that all of the inactive compounds have a substituent on the *ortho*-position of the phenyl ring (the naphthyl ring of **9h** can be regarded as *o*- and *m*-substituted phenyl ring) or a bulkier alkyl group on the α -carbon. Insertion of a methylene group or a hetero atom between the α -carbon and the phenyl ring made the compounds to be inactive (**9i–k** in Figure 21.3). This is apparently due to the electronic effect. The π -electrons of the aromatic ring have an essential effect to promote the reaction.

Putting all the results obtained together, it is expected that introduction of fluorine atoms in the substrate would bring about a favorable effect on the reaction, because fluorine is strongly electron-withdrawing and not so bulky. The results are summarized in Table 21.2 [8]. α -Fluorinated malonate (**9l**) gave the corresponding optically active monobasic acid in a moderate yield. The effect of substitution of ring hydrogens showed again a marked difference between *ortho*- and *meta*- or *para*-positions. Even the steric bulkiness of a fluorine atom has a serious effect on the rate of reaction. The yield of the product after a 5 d reaction of *o*-fluorinated compound **9m** was as low as 12%. The low ee value of the product is probably due to nonenzymatic decarboxylation, which will give a racemic monobasic acid. As is seen from Table 21.2, the *m*- and *p*-trifluoromethyl derivatives (**9p,q**) were good substrates, the chemical and optical yields of the expected products being very high. This can be attributed to the strong electron-withdrawing effect of these substituents.

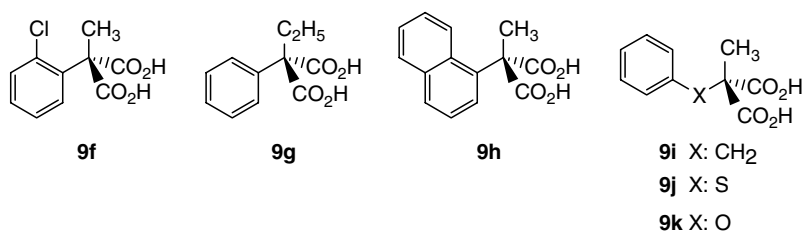


FIGURE 21.3 Inactive compounds to *Alcaligenes bronchisepticus*.

TABLE 21.2
Reaction of Fluorine-Containing Compounds

Compound	Ar	R	Sub. conc. (%)	Yield (%)	ee (%)
9l		F	0.1	64	95
9m		CH ₃	0.3	12	54
9n		CH ₃	0.5	75	97
9o		CH ₃	0.5	54	97
9p		CH ₃	0.5	99	>95
9q		CH ₃	0.5	91	95

21.2.3 PURIFICATION AND CHARACTERIZATION OF THE ENZYME

Although the steric bulkiness around the reaction center is rather restricted, the enzyme system of *A. bronchisepticus* was proved to have a unique reactivity. Thus, detailed studies on the isolated enzyme were expected to elucidate some new interesting mechanism of the new type of decarboxylation.

The bacterium was grown in a medium containing phenylmalonate as the enzyme inducer. The decarboxylation enzyme was purified according to the standard protocol. Column chromatography was performed using three kinds of gels after fractionation by ammonium sulfate. The enzyme was purified to about 300-fold to 377 U/mg protein (Table 21.3). SDS-PAGE and HPLC analysis showed that this enzyme was monomeric, its molecular mass being about 24 kDa (Table 21.4). The enzyme was named arylmalonate decarboxylase (AMDase), as the reaction rate of enzyme-catalyzed decarboxylation of phenylmalonate is faster than that of the α -methyl derivative [9].

To clarify the characteristics of the enzyme, the effects of additives were examined using phenylmalonate as the representative substrate [9]. The addition of ATP and coenzyme

TABLE 21.3
Purification of the Enzyme

Purification	Total Protein (mg)	Total Activity	Specific Activity (U/mg)	Yield (%)
Cell-free extract	8630	10950	1.26	100
Heat treatment	4280	9540	2.22	87
Ammonium sulfate	2840	10350	3.64	95
DEAE-Toyopearl	244	5868	24.1	54
Butyl-Toyopearl	14.7	3391	231	31
QAE-Toyopearl	4.32	1627	377	15

TABLE 21.4
Property of the Enzyme

Molecular Weight	
Gel filtration	22,000
SDS-PAGE	24,000
Number of subunits	1
Stability	
pH	7.0–8.5
Temperature	<40°C
Optimum	
pH	8.5 (Tris-HCl Buffer)
Temperature	45°C

A (CoA) to the enzyme reaction mixtures did not enhance the rate of reaction. In the case of malonyl-CoA decarboxylase and others, ATP and substrate acid form a mixed anhydride, which in turn reacts with CoA to form a thiol ester of the substrate. In the present case, as both ATP and CoA-SH had no effect, the normal mechanism is very unlikely to operate. It is well established that avidin is a potent inhibitor of the formation of the biotin–enzyme complex [10–13]. In this case, addition of avidin had no influence on the decarboxylase activity, indicating that AMDase is not a biotin enzyme (Table 21.5). Thus, the cofactor requirements of AMDase are entirely different from those of known analogous enzymes, such as acyl-CoA carboxylases [14], methylmalonyl-CoA decarboxylases [10], and transcarboxylases [14,15].

A strong inhibitory effect on AMDase activity was found for sulfhydryl reagents (at 1 mM), such as HgCl₂ (relative activity, 0%), HgCl (8%), AgNO₃ (3%), iodoacetate (3%), and *p*-chloromercuribenzoate (PCMB) (0%) (Table 21.5). *N*-Ethylmaleimide (at 10 mM) causes 72% inhibition of the decarboxylase activity. Thus, AMDase was revealed to be a thiol decarboxylase, i.e., at least one of the cysteine residues is present as a free SH that plays an essential role in the active site of the enzyme. The activity of the enzyme was not lost upon incubation with the following reagents: several divalent metal cations such

TABLE 21.5
Effect of Additives

Inhibitor	Concentration (mM)	Relative Activity (%)
None	—	100
NiCl ₂ , MnCl ₂ , CoCl ₂	10	90–96
BaCl ₂ , MgCl ₂ , CaCl ₂	1	17–18
SnCl ₂ , PbCl ₂	1	0
HgCl ₂		
HgCl	1	8
AgNO ₃	1	3
DNB, PCMB, Iodoacetate	1	0–3
<i>N</i> -ethylmaleimide	1	91
PMSF	10	85
NaN ₃ , NH ₂ OH, KCN	10	103–108
EDTA, Bipyridil	10	103–108
Avidin	2.5 mg/ml	99

as Ni^{2+} , Co^{2+} , Ba^{2+} , Mg^{2+} , and Ca^{2+} ; carbonyl reagents such as NaN_3 , NH_2OH , and KCN ; metal-chelating agents such as EDTA, 8-quinolinol, bipyridil, and 1,10-phenanthroline; serine inhibitors such as phenylmethanesulfonyl fluoride (at 10 mM); and others. It is concluded that AMDase is an unusual enzyme which does not contain metal ions or coenzymes that are present in decarboxylases and transcarboxylases.

21.2.4 CLONING OF AMDASE GENE

In order to obtain large amounts of the enzyme, we evaluated the cloning and overexpression of AMDase gene using the direct expression method [16]. The genomic DNA isolated from *A. bronchisepticus* was completely digested by *Pst*I and cloned into pUC19 *Pst*I site. *Escherichia coli* DH5 α -MCR was transformed by the plasmids, and the genomic libraries were constructed. The transformants were screened by the change of color of the plate containing bromothymol blue and phenylmalonic acid, due to the local change of pH of the medium around the active colony as a result of the formation of monobasic acid from the starting dibasic acid. One in approximately 700 transformants exhibited AMDase activity. The plasmid (pAMD100) contained an insert of about 2.8 kbp and was sequenced. The gene was revealed to be consisting of 720 bp, which means that the enzyme consists of 240 amino acids (Figure 21.4). A *Pst*I–*Hind*III (1.2 kbp) fragment was subcloned in pUC19 to generate

```

                                30                                60
at g cag caa gca agc act ccc acc at c ggc at g at c gt g ccg ccc gcc gcg ggt ct g gt g
Met Gln Gln Ala Ser Thr Pro Thr Ile Gly Met Ile Val Pro Pro Ala Ala Gly Leu Val
                                90                                120
ccg gcg gat ggg gcg cgg ct c tat ccc gat ct g ccc ttc att gcc agc ggg ct g ggg ct g
Pro Ala Asp Gly Ala Arg Leu Tyr Pro Asp Leu Pro Phe Ile Ala Ser Gly Leu Gly Leu
                                150                                180
ggc tcc gt c acg ccg gaa ggc tat gac gcc gt g at c gaa tcc gt g gt g gac cat gcg cgc
Gly Ser Val Thr Pro Glu Gly Tyr Asp Ala Val Ile Glu Ser Val Val Asp His Ala Arg
                                210                                240
cgc ct g caa aag cag ggc gcg gcg gt g gtt tcc ct g at g gcc acc tcc ct c agc ttc tac
Arg Leu Gln Lys Gln Gly Ala Ala Val Val Ser Leu Met Gly Thr Ser Leu Ser Phe Tyr
                                270                                300
cgg ggc gcg gcc ttc aat gcc gcg ttg acc gta gcg at g ccg gaa gcc acg gga ct g cca
Arg Gly Ala Ala Phe Asn Ala Ala Leu Thr Val Ala Met Arg Glu Ala Thr Gly Leu Pro
                                330                                360
tgc acg acc at g agc acg gcg gt c ct g aac gga ttg ccg gcc ct g gcc gt g ccg gcg gt c
Cys Thr Thr Met Ser Thr Ala Val Leu Asn Gly Leu Arg Ala Leu Gly Val Arg Arg Val
(101)
                                390                                420
gcg ttg gcg acg gcc tat at c gac gat gt g aac gag ccg ct g gcg gca ttc ct g gcc gaa
Ala Leu Ala Thr Ala Tyr Ile Asp Asp Val Asn Glu Arg Leu Ala Ala Phe Leu Ala Glu
                                450                                480
gag agc ct g gtt ccc acc ggc tgc ccg agc ctt ggc at c acg gcc gt g gag gcc at g gcg
Glu Ser Leu Val Pro Thr Gly Cys Arg Ser Leu Gly Ile Thr Gly Val Glu Ala Met Ala
(148)
                                510                                540
cgc gt g gat acg gcc acg ct g gt c gac ct g tgc gt g cgt gcc ttc gaa gcg gcg ccc gat
Arg Val Asp Thr Ala Thr Leu Val Asp Leu Cys Val Arg Ala Phe Glu Ala Ala Pro Asp
(171)
                                570                                600
agc gac gcc at c ct g ct g tct tgc gcc ggc ttg ct g acg ct g gac gcc ata ccc gaa gt c
Ser Asp Gly Ile Leu Leu Ser Cys Gly Gly Leu Leu Thr Leu Asp Ala Ile Pro Glu Val
(188)
                                630                                660
gag ccg ccg ct g gcc gt g ccg gt g gt g tcc agt tcc ccg gcg ggg ttc tgg gac gcc gt g
Glu Arg Arg Leu Gly Val Pro Val Val Ser Ser Ser Pro Ala Gly Phe Trp Asp Ala Val
                                690                                720
cgg ctt gcg ggg gga ggg gcc aag gca agg ccc gga tac gcc ccg ct g ttc gac gag tcc
Arg Leu Ala Gly Gly Gly Ala Lys Ala Arg Pro Gly Tyr Gly Arg Leu Phe Asp Glu Ser
tga
***

```

FIGURE 21.4 Nucleotide and deduced amino acid sequences of the AMDase.

pAMD101. The amount of the enzyme in a cell-free extract of the transformant, *E. coli* JM109/pAMD101, was elevated to 37,250 units/L culture broth. It was calculated that the enzyme comprised over 25% of the total extractable cellular proteins. The enzyme produced by the *E. coli* transformant was purified to homogeneity and shown to be identical to that of the original strain. Both enzymes had the same enzymological properties and N-terminal amino acid sequences.

21.2.5 SITE-DIRECTED MUTAGENESIS OF AMDASE GENE

DNA sequence indicated that AMDase contains four cysteine residues located at 101, 148, 171, and 188 from the amino terminal (Figure 21.4) [16]. At least one of these four is estimated to play a crucial role in the decarboxylation of disubstituted malonic acid. The most direct method to locate which Cys is responsible for enzyme activity is site-directed mutagenesis. Then, we have to ascertain which mutation is effective for this purpose. To determine the amino acid that should be introduced in place of Cys, we have to consider the role of the cysteine residue. One possibility is that it works as a nucleophile. Attack on the carbonyl carbon will result in the thiol ester, which will stabilize the enolate-type transition state in place of CoA. Enantioselective protonation followed by hydrolysis at the final step will give (*R*)- α -arylpropionate. SH group can work as an acid. Partial protonation to α -carbon will facilitate the C–C bond fission and accelerate the reaction. Formation of the C–H bond gives the observed optically active product. Then, from the standpoint of organic reaction mechanism, substitution of the sulfur atom with an oxygen atom would greatly decrease the rate of reaction, because nucleophilicity, anion-stabilizing effect, and proton-donating ability of a hydroxyl group are far smaller than those of an SH group. Still, a hydroxyl group is capable of more or less keeping the hydrogen bondings in which the cysteine residues are incorporated. Accordingly, the activity of enzyme was expected to remain partly, whichever Cys is replaced by Ser, different from the cases in which alanine or some other totally different amino acid residue is introduced in place of Cys. Thus, four mutant genes in which one of the four codons of Cys is replaced by that of Ser were prepared and expressed in *E. coli*. Four mutant enzymes were isolated, purified, and incubated with phenylmalonate (**7**) [17]. Kinetic data for the mutant enzymes and the wild enzyme are summarized in Table 21.6. Among the four mutants, C188S showed a drastic decrease in activity (k_{cat}/K_m). This low activity was due to a decrease in the catalytic turnover number (k_{cat}) rather than an affinity to the substrate (K_m).

The CD spectrum of the C188S mutant is essentially the same as that of the wild-type enzyme, which reflects that the tertiary structure of this mutant changed little compared to that of the wild-type enzyme. Calculation of the content of the secondary structure of mutant enzymes based on J-600S Secondary Structure Estimation system (JASCO) also showed that there is no significant change in the tertiary structure of the C188S mutant. The fact that the k_{cat} value of this mutant is extremely small despite little change in conformation clearly

TABLE 21.6
Reactivities of Wild Type and Mutant Enzymes

	K_m (mM)	k_{cat} (S^{-1})	k_{cat}/K_m
Wild type	13.3	366	27.5
C101S	4.3	248	57.6
C148S	11.5	100	8.7
C171S	9.1	62.3	6.8
C188S	4.9	0.62	0.13

indicates that Cys188 is located in the active site. The catalytic activity of mutants C148S and C171S also decreased in spite of the smaller K_m values compared to that of the wild-type enzyme. It can be assumed that the decrease in α -helix structure caused a decrease in k_{cat} value. The distance between the catalytic amino acid and the binding substrate would become longer because of the change in conformation. It was thus concluded that cysteine188 is located in the catalytic site of the enzyme.

21.2.6 STEREOCHEMICAL COURSE OF THE REACTION

One can ask whether the enzyme distinguishes between two prochiral carboxyl groups? The clue to elucidation of this question is to prepare both enantiomers of α -methyl- α -phenylmalonate that have ^{13}C on one of the two carboxyl groups. Starting from ^{13}C -phenylacetate (^{13}C -**11**), by optical resolution of an intermediate **14**, both enantiomers of chiral ^{13}C -containing α -methyl- α -phenylmalonate were prepared (Figure 21.5). The absolute configuration of the chiral substrate was unambiguously determined by the optical rotation of the resolved hydroxyl acid **14**.

The result of enzymatic decarboxylation was very clear [18]. While (*S*)-**15** resulted in ^{13}C containing product (**12**), (*R*)-**15** gave the product with ^{13}C no more than natural abundance. Apparently the reaction proceeds with net inversion of configuration. Thus, the presence of a planar intermediate, such as **21**, can be postulated. Enantioface-differentiating protonation to **21** will give the optically active final product. One evidence supporting this intermediate is the

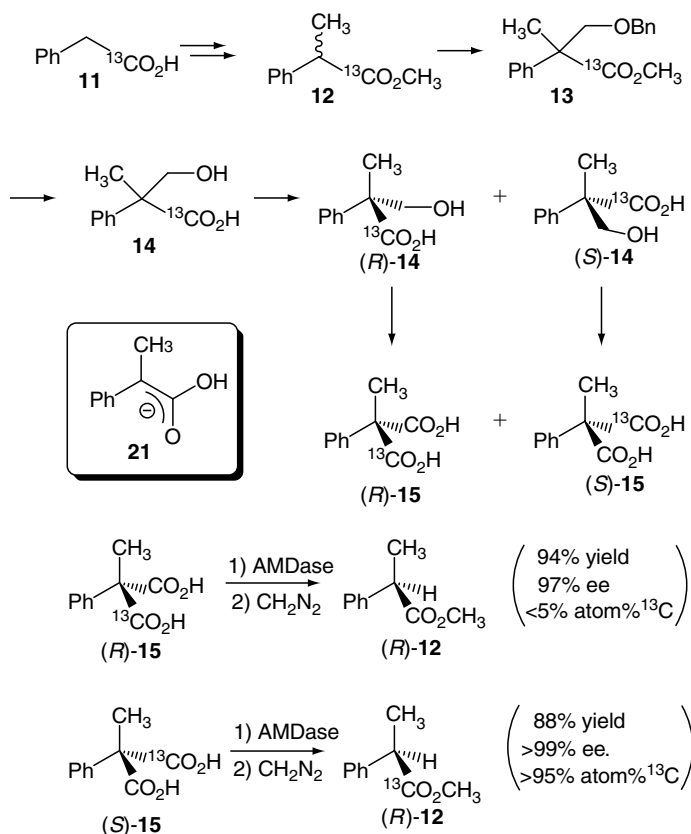


FIGURE 21.5 Stereochemistry of AMDase-catalyzed decarboxylation.

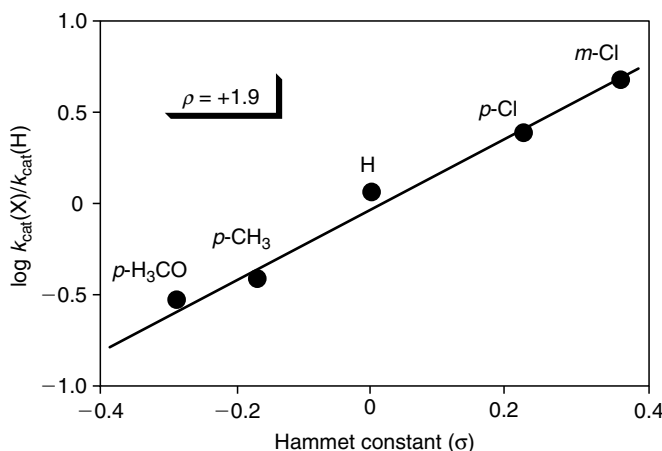


FIGURE 21.6 Hammett plot of the k_{cat} for the AMDase-catalyzed decarboxylation of series of X-phenylmalonates.

electronic effect of the substituents on the rate of decarboxylation of substituted (*p*-MeO, *p*-Me, *p*-Cl, *m*-Cl, and H) phenylmalonic acid. The logarithm of $k_{\text{cat}}(\text{X})/k_{\text{cat}}(\text{H})$ cleanly correlated in a linear fashion to Hammett σ -values (Figure 21.6). The ρ -value was revealed to be +1.9. The fact that the sign of the ρ -value is positive means that the transition state has some negative charge. Thus, the supposition of a negatively charged intermediate such as **21** is rationalized. In this case, the aromatic ring should occupy the same plane as that of the olefinic part. It is then estimated that the conformation of the substrate is already restricted when it binds to the active site of the enzyme.

As the conformation of the intermediate **21** reflects the one of the starting materials, *ortho*-substituents (X) and α -substituent (CH₃) should be arranged in *syn*- or *anti*-periplanar. As mentioned earlier (Figure 21.7), *ortho*-substituted compounds (X = Cl, CH₃) and α -ethyl derivative are inactive to this enzyme in contrast to substrates, in which either methyl or X is replaced by a hydrogen, that is readily decarboxylated by the enzyme. The difference in reactivities of these compounds is well deduced by supposing that the substrates are required to take the *syn*-periplanar conformation to bind to the enzyme or to undergo decarboxylation reaction. Accordingly, if the loss of potential energy to take the *syn*-periplanar conformation exceeds the binding energy between the substrate and the enzyme, the compound will be inactive. The K_m values of ordinary substrates are 10 to 20 mM, indicating that the binding energy is about 2.5 to 3 kcal/mol at the reaction temperature. Then, how much is the difference of potential energies between *syn*-periplanar conformation and the most stable conformation? As there are no experimental data available, the values were obtained by *ab initio* calculation. The results were clear. The difference in potential energy between *syn*- and *anti*-periplanar conformation (the most stable conformation) of α -methyl- α -(*o*-chlorophenyl)malonate (**9f**) is calculated to be 5.5 kcal/mol, whereas that of the nonmethylated compound is about 0.7 kcal/mol. These values together with the binding energy of reactive substrates demonstrate the essential importance of conformation of the substrate in the pocket of the enzyme [19]. At least one of the attracting forces to fix the conformation of the substrates will be CH- π interaction between the aromatic rings of the enzyme and the substrate [20].

21.3 CONVERSION OF THE FUNCTION BY RATIONAL DESIGN

21.3.1 INVERSION OF ENANTIOSELECTIVITY OF AMDASE

Some enzymes were found that had about 30% homology and some common functions through multiple alignments using the PSI-BLAST program. These were glutamate racemase from *Lactobacillus fermenti* [22], aspartate racemase from *Streptococcus thermophilus* [23], hydantoine racemase from *Pseudomonas* sp. strain NS671 [24], and maleate isomerase from *A. faecalis* [25]. The important feature that is consistent for all these enzymes is the presence of Cys188. While all the racemases have another cysteine residue at around 74, AMDase has no corresponding cysteine residue around this region as shown in Figure 21.8.

[illegible]

© 2006 by Taylor & Francis Group, LLC.

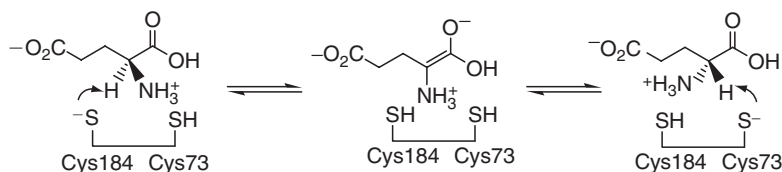


FIGURE 21.9 Reaction mechanism of glutamate racemase.

Thus, one cysteine residue abstracts the α -proton from the substrate, while the other delivers a proton from the opposite side of the intermediate enolate of the amino acid. In this way, the racemase catalyzes the racemization of glutamic acid through a so-called two-base mechanism (Figure 21.9).

The tertiary structure of glutamate racemase has already been resolved, and it has also been clarified that a substrate analog glutamine binds between two cysteine residues [29]. These data enabled us to predict that the new proton-donating amino acid residue should be introduced at position 74, replacing Gly for the inversion of enantioselectivity of the decarboxylation reaction.

First, we examined the enantioselectivity of a C188S mutant enzyme purified from *E. coli* JM109/pAMD101 (Table 21.8). Although, the proton-donating ability of Ser is weaker than Cys, the location of the proton donor does not change in this case. Thus, we presumed that the configuration of the product would be the same as in the case of the reaction by the wild-type enzyme. To our surprise, however, the results were entirely different: α -methyl- α -thienyl (**9e**) and α -methyl- α -naphthylmalonate (**20**) gave the corresponding monobasic acids with the configuration opposite to that given by the native enzyme (Table 21.8). This fact suggests that there are some other proton donors on the opposite side of the enantiomeric face of the intermediate enolate, although their effect is far smaller compared with Cys188. In the case of

TABLE 21.8
Enantioselectivities of Mutant Enzymes

Enzyme	Ar	Yield (%)	ee (%)
Wild type		quant	99 (<i>S</i>)
C188S		17	50 (<i>R</i>)
G74C		37	0 (–)
G74C/C188S		60	94 (<i>R</i>)
Wild type		96	97 (<i>R</i>)
C188S		6	70 (<i>S</i>)
G74C		13	6 (<i>R</i>)
G74C/C188S		17	96 (<i>S</i>)

the C188S mutant, as the proton-donating ability of serine is weaker than that of cysteine, the hidden effect of the other proton donors might be reflected in the product. Then, higher ee will be attained if the Cys188 of the native enzyme is changed by an amino acid that has no acidic proton. Thus, we prepared the C188A mutant. However, this mutant had no enzyme activity and, in addition, was very unstable. This means that a Cys or Ser residue is inevitable at position 188, and is probably involved in a hydrogen bonding. Therefore, we decided to introduce a Cys residue as a proton donor instead of the Gly74 of the native enzyme.

The G74C mutant was prepared by polymerase chain reaction (PCR) using the plasmid that contains the gene coding native AMDase (pAMD101). The amplified gene was digested by *Hind*III and *Pst*I followed by ligation with pUC19. The mutant enzyme was purified from the transformed *E. coli* cells. Although the change in amino acid is drastic, the mutant still exhibited some activity. As expected, the products were nearly racemic, if not entirely, in the case of both **9e** and **20** (Table 21.8). These results demonstrate that this position is effective to give a proton to the intermediate of the reaction.

If the proton-donating ability of the amino acid at 188 is weaker, the enantioselectivity of the reaction is expected to become the opposite. So we prepared a double mutant G74C/C188S gene starting from the one that already contains the codon for C188S mutation. As shown in Table 21.8, the absolute configuration of the products is opposite to that of the products obtained by the native enzyme, and the ee of the products dramatically increased to 94% and 96% for **9e** and **20**, respectively (Table 21.8). This inversion of the enantioselectivity of the reaction supports the reaction mechanism where the Cys188 of the native enzyme is working as the proton donor.

REFERENCES

1. Kim, Y.S. and Kolattukudy, P.E., Stereospecificity of malonyl-CoA decarboxylase, acetyl-CoA carboxylase, and fatty acid synthetase from the uropygial gland of goose, *J. Biol. Chem.*, 255(2), 686–689, 1980.
2. Thomas, N.R., Schirch, V., and Gani, D., Synthesis of (2*R*)- and (2*S*)-[1–¹³C]-2-amino-2-methylmalonic acid, probes for the serine hydroxymethyltransferase reaction: stereospecific decarboxylation of the 2-pro-*R* carboxy group with the retention of configuration, *J. Chem. Soc. Chem. Commun.*, 5, 400–402, 1990.
3. Thomas, N.R., Rose, J.E., and Gani, D., Decarboxylation of 2-aminomalonic acid catalysed by serine hydroxymethyltransferase is, in fact, a stereospecific process, *J. Chem. Soc. Chem. Commun.*, 14, 908–909, 1991.
4. Harrison, I.T., Lewis, B., Nelson, P., et al., Nonsteroidal anti-inflammatory agents. I. 6-Substituted 2-naphthylacetic acids, *J. Med. Chem.*, 13(2), 203–205, 1970.
5. Shen, T.Y., Perspectives in nonsteroidal anti-inflammatory agents, *Angew. Chem. Int. Ed. Engl.*, 11(6), 460–472, 1972.
6. Miyamoto, K. and Ohta, H., Enzyme-mediated asymmetric decarboxylation of disubstituted malonic acids, *J. Am. Chem. Soc.*, 112, 4077–4078, 1990.
7. Miyamoto, K. and Ohta, H., Asymmetric decarboxylation of disubstituted malonic acid by *Alcaligenes bronchisepticus* KU1201, *Biocatalysis*, 5, 49–60, 1991.
8. Miyamoto, K., Tsuchiya, S., and Ohta, H., Microbial asymmetric decarboxylation of fluorine-containing arylmalonic acid derivatives, *J. Fluorine Chem.*, 59, 225–232, 1992.
9. Miyamoto, K. and Ohta, H., Purification and properties of a novel arylmalonate decarboxylase from *Alcaligenes bronchisepticus* KU1201, *Eur. J. Biochem.*, 210, 475–481, 1992.
10. Galivan, J.H. and Allen S.H.G., Methylmalonyl-CoA decarboxylase: partial purification and enzymatic properties, *Arch. Biochem. Biophys.*, 126(3), 838–847, 1968.
11. Hoffmann, A., Hilpert, W., and Dimroth, P., The carboxyltransferase activity of the sodium-ion-translocating methylmalonyl-CoA decarboxylase of *Veillonella alcalescens*, *Eur. J. Biochem.*, 179(3), 645–650, 1989.

12. Green, N.M., A spectrophotometric assay for avidin and biotin based on binding of dyes by avidin, *Biochem. J.*, 94(3), 23c–24c, 1965.
13. Green N.M. and Toms E.J., Purification and crystallization of avidin, *Biochem. J.*, 118(1), 67–70, 1970.
14. Boyer, P.D., Ed., *The Enzymes*, vol. 6: *Carboxylation and Decarboxylation (Nonoxidative)*, Academic Press, New York, 1972, pp. 39–115.
15. Wood, H.G., Lochmueller, H., Riepertinger, C., et al., Transcarboxylase. IV. Function of biotin and the structure and properties of the carboxylated enzyme, *Biochem. Z.*, 337, 247–266, 1963.
16. Miyamoto, K. and Ohta, H., Cloning and heterologous expression of a novel arylmalonate decarboxylase gene from *Alcaligenes bronchisepticus* KU1201, *Appl. Microbiol. Biotechnol.*, 38, 234–238, 1992.
17. Miyazaki, M., Kakidani, H., Hanzawa, S., et al., Cysteine188 revealed as being critical for the enzyme activity of arylmalonate decarboxylase by site-directed mutagenesis, *Bull. Chem. Soc. Jpn.*, 70(11), 2765–2769, 1997.
18. Miyamoto, K., Tsuchiya, S., and Ohta, H., Stereochemistry of enzyme-catalyzed decarboxylation of α -methyl- α -phenylmalonic acids, *J. Am. Chem. Soc.*, 114, 6256–6257, 1992.
19. Miyamoto, K., Ohta, H., and Osamura, Y., Effect of conformation of the substrate on enzymatic decarboxylation of α -arylmalonic acid, *Bioorg. Med. Chem.*, 2, 469–475, 1994.
20. Kawasaki, T., Saito, K., and Ohta, H., The mode of substrate-recognition mechanism of arylmalonate decarboxylase, *Chem. Lett.*, 26(4), 351–352, 1997.
21. Kawasaki, T., Horimai, E., and Ohta, H., On the conformation of the substrate binding to the active site during the course of enzymic decarboxylation, *Bull. Chem. Soc. Jpn.*, 69(12), 3591–3594, 1996.
22. Gallo, K.A. and Knowles, J.R., Purification, cloning, and cofactor independence of glutamate racemase from *Lactobacillus*, *Biochemistry*, 32(15), 3981–3990, 1993.
23. Yohda, M., Okada, H., and Kumagai, H., Molecular cloning and nucleotide sequencing of the aspartate racemase gene from lactic acid bacteria *Streptococcus thermophilus*, *Biochim. Biophys. Acta*, 1089(2), 234–240, 1991.
24. Watabe, K., Ishikawa, T., Mukohara, Y., et al., Identification and sequencing of a gene encoding a hydantoin racemase from the native plasmid of *Pseudomonas* sp. strain NS671, *J. Bacteriol.*, 174, 3461–3466, 1992.
25. Hatakeyama, K., Asai, Y., Uchida, Y., et al., Gene cloning and characterization of maleate *cis-trans* isomerase from *Alcaligenes faecalis*, *Biochem. Biophys. Res. Commun.*, 239(1), 74–79, 1997.
26. Glavas, S. and Tanner, M.E., Catalytic acid/base residues of glutamate racemase, *Biochemistry*, 38(13), 4106–4113, 1999.
27. Gallo, K.A., Tanner, M.E., and Knowles, J.R., Mechanism of the reaction catalyzed by glutamate racemase, *Biochemistry*, 32(15), 3991–3997, 1993.
28. Tanner, M.E., Gallo, K.A., and Knowles, J.R., Isotope effects and the identification of catalytic residues in the reaction catalyzed by glutamate racemase, *Biochemistry*, 32(15), 3998–4006, 1993.
29. Hwang, K.Y., Cho, C.-S., Kim, S.S., et al., Structure and mechanism of glutamate racemase from *Aquifex pyrophilus*, *Nat. Struct. Biol.*, 6, 422–426, 1999.

22 Chemoenzymatic Preparation of Enantiopure Building Blocks of Synthetic Utility

Kenji Mori

CONTENTS

22.1	Introduction	564
22.2	Desymmetrization of Acyclic <i>Meso</i> -Diols with Esterases and Lipases	565
22.2.1	(2 <i>R</i> ,4 <i>S</i>)-5-Acetoxy-2,4-Dimethyl-1-Pentanol (2)	565
22.2.1.1	Preparation of (2 <i>R</i> ,4 <i>S</i>)- 2	565
22.2.2	(2 <i>R</i> ,6 <i>S</i>)-7-Acetoxy-2,6-Dimethyl-1-Heptanol (4)	566
22.2.2.1	Preparation of (2 <i>R</i> ,6 <i>S</i>)- 4	566
22.2.3	(1 <i>S</i> ,2 <i>R</i>)-1-Acetoxymethyl-2-Hydroxymethylcyclopropane (6)	567
22.2.3.1	Preparation of (1 <i>S</i> ,2 <i>R</i>)- 6	567
22.2.4	(2 <i>S</i> ,3 <i>R</i>)-4-Acetoxy-2,3-Epoxy-1-Butanol (8)	567
22.2.4.1	Preparation of (2 <i>S</i> ,3 <i>R</i>)- 8	568
22.2.5	(2 <i>S</i> ,3 <i>R</i>)-4-Acetoxy-2,3-Isopropylidenedioxy-1-Butanol (10)	568
22.2.5.1	Preparation of (2 <i>S</i> ,3 <i>R</i>)- 10	568
22.2.6	(<i>R</i>)-2-Acetoxymethyl-3-Buten-1-ol (12)	569
22.3	Asymmetric Reduction of Prochiral Ketones with Yeasts	570
22.3.1	(<i>S</i>)-1-Phenylthio-2-Butanol (14)	570
22.3.1.1	Preparation of (<i>S</i>)- 14	570
22.3.2	(<i>S</i>)-3-Hydroxy-2,2-Dimethylcyclohexanone (17)	571
22.3.2.1	Preparation of (<i>S</i>)- 17	571
22.3.3	(1 <i>R</i> ,4 <i>S</i> ,6 <i>S</i>)-6-Hydroxy-1-Methylbicyclo[2.2.2]octan-2-one (19)	573
22.3.3.1	Preparation of (1 <i>R</i> ,4 <i>S</i> ,6 <i>S</i>)- 19	573
22.3.4	Other 3-Hydroxy Ketones	573
22.3.4.1	Preparation of (1 <i>R</i> ,4 <i>S</i> ,6 <i>S</i>)- 25	574
22.3.5	Octahydronaphthalenone (28) and Octahydroindenone (31)	575
22.3.6	Cyclic and Enolizable β -Keto Esters with a Single Stereogenic Center at α -Position	575
22.3.6.1	Preparation of (1 <i>R</i> ,2 <i>S</i>)- 33	576
22.3.6.2	Preparation of (1 <i>R</i> ,2 <i>S</i>)- 35	576
22.4	Kinetic Resolution of Racemic Alcohols, Hydroxy Acids, and Amino Acids with Hydrolytic Enzymes	577
22.4.1	(2 <i>R</i> ,3 <i>R</i>)-1-Acetoxy-4-(<i>t</i> -Butyldiphenylsilyloxy)-2,3-Epoxybutane (37)	577
22.4.1.1	Preparation of (2 <i>S</i> ,3 <i>R</i>)- 37 and (2 <i>R</i> ,3 <i>S</i>)- 36	577

22.4.2	(2 <i>S</i> ,3 <i>R</i>)-3,7-Dimethyl-2,3-Epoxy-6-Octenyl Acetate (39) and (2 <i>R</i> ,3 <i>S</i>)-3,7-Dimethyl-2,3-Epoxy-6-Octen-1-ol (38).....	578
22.4.2.1	Preparation of (2 <i>S</i> ,3 <i>R</i>)- 39 and (2 <i>R</i> ,3 <i>S</i>)- 38	578
22.4.3	Enantiomers of 2-(2',2'-Dimethyl-6'-Methylenecyclohexyl)ethanol (40)	578
22.4.4	Enantiomers of 2,4,4-Trimethyl-2-Cyclohexen-1-ol (43)	579
22.4.4.1	Preparation of (<i>R</i>)- 43 and (<i>S</i>)- 42	580
22.4.5	(3 <i>aR</i> ,5 <i>S</i> ,8 <i>bS</i>)-5-Hydroxy-8,8-Dimethyl-3,3 <i>a</i> ,4,5,6,7,8, 8 <i>b</i> -Octahydroindeno[1.2- <i>b</i>]furan-2-one (45).....	580
22.4.6	(<i>R</i>)-3-Hydroxy-15-Methylhexadecanoic Acid (47) and (<i>R</i>)-2-Hydroxy-21-Methyldocosanoic Acid (48).....	580
22.4.6.1	Preparation of (<i>R</i>)- 47	580
22.4.7	(2 <i>R</i> ,3 <i>S</i>)- and (2 <i>S</i> ,3 <i>R</i>)-3-Acetoxy-2-(Octadecanoylamino)- 1-Hexadecanol (50)	581
22.4.8	(4 <i>S</i> ,6 <i>S</i>)-4-Hydroxy-4,9-Dioxaspiro[5.5]undecane (52) and (<i>R</i>)-Tetrahydropyranyl Alcohol 53	582
22.4.9	(<i>R</i>)- and (<i>S</i>)-2-Aminohexadecanoic Acid (56).....	582
22.4.9.1	Preparation of the Enantiomers of 56	582
22.5	Kinetic Resolution of Racemic Ketones by Asymmetric Reduction with Yeasts	583
22.5.1	(1 <i>S</i> ,2 <i>R</i> ,5 <i>R</i>)-2-Ethoxycarbonyl-7,7-Ethylenedioxybicyclo [3.3.0]octan-3-one (57) and (1 <i>S</i> ,5 <i>R</i> ,6 <i>R</i> ,7 <i>S</i>)-6-Ethoxycarbonyl-3, 3-Ethylenedioxy-7-Hydroxybicyclo[3.3.0]octane (58).....	583
22.5.2	(<i>R</i>)- and (<i>S</i>)-Wieland–Miescher Ketone (59).....	585
22.5.2.1	Preparation of (<i>R</i>)- 59	585
22.5.2.2	Preparation of (4 <i>aS</i> ,5 <i>S</i>)- 60	585
22.6	Conclusion	586
	Acknowledgment	587
	References	587

22.1 INTRODUCTION

Since 1978 we have been employing hydrolytic enzymes and yeasts as biocatalysts to prepare enantiopure building blocks for our laboratory-scale synthesis of pheromones and terpenes. In this chapter, preparative methods of some enantiopure building blocks will be described together with the experimental details for the preparation of especially useful building blocks. Unlike the traditional industrial use of biocatalysts (“fermentation”) to provide compounds with complicated structures such as antibiotics starting from cane molasses or other carbon sources, the approach detailed in this chapter is to prepare enantiopure building blocks that can eventually be converted by organic reactions to more value-added products like pheromones and terpenes (Figure 22.1). Design of versatile building blocks can be best carried out by synthetic chemists, who know the scope and limitations of various organic reactions to be used in conversion of the building blocks to the target molecules.

There are two choices for the biocatalytic preparation of the enantiopure building blocks. One is the desymmetrization of *meso*-compounds and prochiral compounds, while the other is the kinetic resolution of racemic samples. These two methods will be discussed in detail. It must be added that desymmetrization is more efficient than kinetic resolution. The former converts all of the starting material to the desired product, while the latter gives the desired enantiomer in 50% yield at most. In special cases, such as in the cases of chiral and enolizable β -keto esters with an α -substituent, their enantiomers can interconvert to each other through

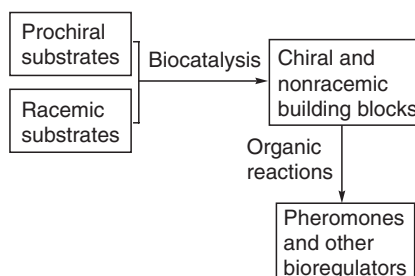


FIGURE 22.1 Roles of biocatalysis and organic reactions in the synthesis of pheromones and other bioregulators.

keto-enol tautomerism, and more readily reducible ones can be reduced with reductases in yeast to give optically active β -hydroxy esters in more than 50% yield. This is an example of dynamic kinetic resolution.

22.2 DESYMMETRIZATION OF ACYCLIC *MESO*-DIOLS WITH ESTERASES AND LIPASES

22.2.1 (2*R*,4*S*)-5-ACETOXY-2,4-DIMETHYL-1-PENTANOL (**2**)

The compound **2** is a useful building block for the synthesis of 1,3-dimethylated alkyl chains of natural products, such as (2*R*,4*R*)-supellapyrone, the female-produced sex pheromone of the broad-banded cockroach, *Supella longipalpa*. Asymmetric acetylation of diol **1** with vinyl acetate and lipase AK (Amano) gives (2*R*,4*S*)-**2** (98.0% ee) in 72% yield (Figure 22.2) [1]. In order to find out the optimal reaction conditions, it is necessary to screen lipases and esterases, solvents [usually tetrahydrofuran (THF), diisopropyl ether, or diethyl ether], acyl donors (usually vinyl acetate or isopropenyl acetate), and reaction temperature.

22.2.1.1 Preparation of (2*R*,4*S*)-**2** [1]

Lipase AK (250 mg) was added to a cooled and stirred solution of **1** (5.18 g, 39.2 mmol) in THF (50 mL) at 0°C. Vinyl acetate (3.7 mL, 43 mmol) was added to the mixture, which was stirred for 3 d at 5°C. Vinyl acetate (1.5 mL, 17 mmol) was added again, and the mixture was stirred for 2 d, filtered through Celite, and the filtrate was concentrated *in vacuo*. The residue was chromatographed on SiO₂ (50 g, hexane/EtOAc, 20:1) to give (2*R*,4*S*)-**2** (4.95 g, 72%) as an

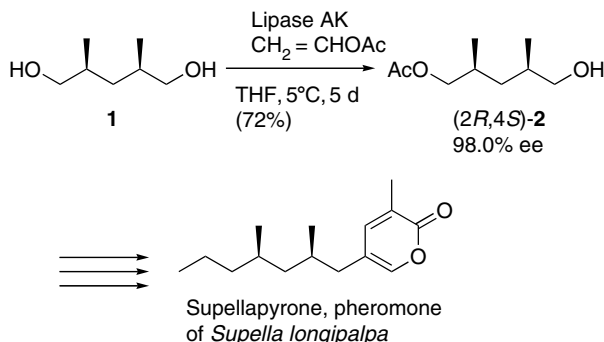


FIGURE 22.2 Preparation and utilization of half-acetate (2*R*,4*S*)-**2**.

oil, $n_D^{25} = 1.4378$; $[\alpha]_D^{25} = +10.6$ ($c = 1.88$, CHCl_3). Enantiomeric purity determination: high-performance liquid chromatography (HPLC) analysis on Chiralcel OD (hexane/EtOH, 30:1), 98.0% ee.

22.2.2 (2*R*,6*S*)-7-Acetoxy-2,6-dimethyl-1-heptanol (4)

1,5-Dimethylated alkyl chain is the characteristic feature of isoprenoids and some pheromones. Asymmetric acetylation of diol **3** with isopropenyl acetate and immobilized lipase PS (Amano) affords (2*R*,6*S*)-**4** (95% ee) in 57% yield (Figure 22.3) [2]. This acetylation was first reported by Chênevert and Desjardins [3]. Several pheromones, as shown in Figure 22.3, and tribolure [3] were synthesized from (2*R*,6*S*)-**4** [2,4,5].

22.2.2.1 Preparation of (2*R*,6*S*)-**4** [2]

Immobilization of Lipase PS: Lipase PS (Amano, 3.0 g) was mixed thoroughly with Hyflo Super Cel (10.0 g). Then, 0.1 M potassium phosphate buffer (pH 7, 10 mL) was added. The mixture was shaken vigorously and dried *in vacuo*.

Acetylation: Immobilized lipase PS (2.20 g) and isopropenyl acetate (6 mL) were added to a solution of **3** (5.30 g, 33.1 mmol) in THF (350 mL). The mixture was stirred at 0°C and

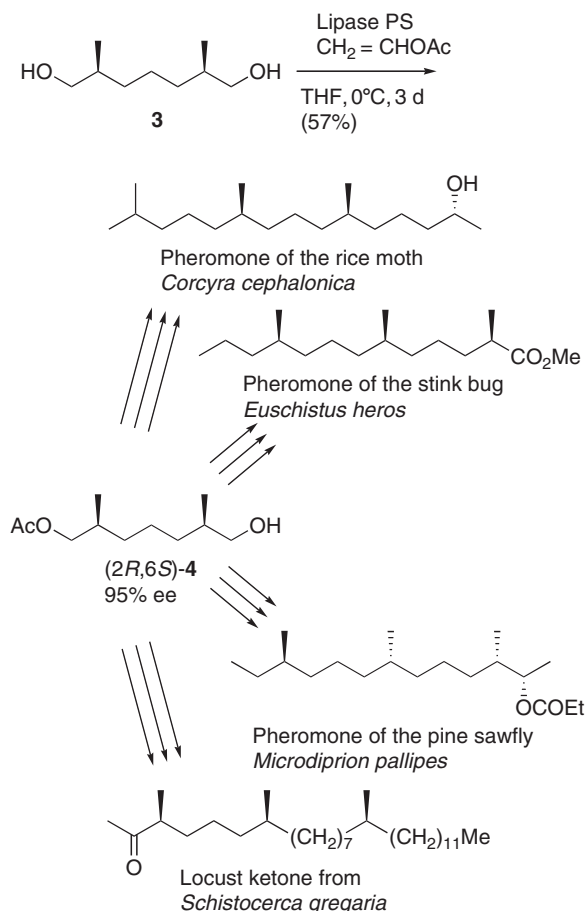


FIGURE 22.3 Preparation and utilization of half-acetate (2*R*,6*S*)-**4**.

monitored by TLC (3d). The solid was filtered off and washed with THF, and the filtrate was concentrated *in vacuo*. The residue was chromatographed on SiO₂ (140 g, hexane/EtOAc 20:1 to 10:1) to give 3.8 g (57%) of **4** as a colorless oil along with diacetate (2.1 g, 26%) and diol (0.7 g, 13%). Properties of **4**: $n_D^{24} = 1.4426$; $[\alpha]_D^{24} = +8.8$ ($c = 0.98$, CHCl₃). Enantiomeric purity determination: derivatization followed by HPLC analysis, 95% ee.

22.2.3 (1*S*,2*R*)-1-Acetoxyethyl-2-Hydroxyethylcyclopropane (**6**)

Cyclopropane-containing fatty acids are occasionally found as natural products. Plakoside A (Figure 22.4) is an immunosuppressive galactosphingolipid isolated from the Caribbean sponge *Plakortis simplex*, and contains two cyclopropane rings. Synthesis and stereochemical assignment of plakoside A were achieved by employing (1*S*,2*R*)-**6** as the key building block [6,7]. As shown in Figure 22.4, acetylation of diol **5** with vinyl acetate and lipase AK (Amano) gives (1*S*,2*R*)-**6** (>99.9% ee) in 86% yield [6]. Asymmetric hydrolysis of a diester of **5** was also reported by others ([8] and references therein), and the resulting optically active mono ester served as a starting material in pheromone synthesis [8].

22.2.3.1 Preparation of (1*S*,2*R*)-**6** [6]

Lipase AK (Amano, 1.06 g) was added to a solution of **5** (21.3 g, 208 mmol) in THF (110 mL) and vinyl acetate (130 mL), and the mixture was stirred for 3.5 h at room temperature. The mixture was filtered through Celite and the filtrate was concentrated *in vacuo*. The residue was chromatographed on SiO₂ to give (1*S*,2*R*)-**6** as a colorless oil (25.8 g, 86%), $n_D^{25} = 1.4558$; $[\alpha]_D^{21} = -19.9$ ($c = 1.65$, CHCl₃). Enantiomeric purity determination: HPLC analysis on Chiralcel OD-H, >99.9% ee.

22.2.4 (2*S*,3*R*)-4-Acetoxy-2,3-Epoxy-1-Butanol (**8**)

Epoxides are frequently found among insect pheromones. Asymmetric hydrolysis of *meso*-diacetate **7** with pig pancreatic lipase (PPL) gives (2*S*,3*R*)-**8** (90% ee) in 71% yield

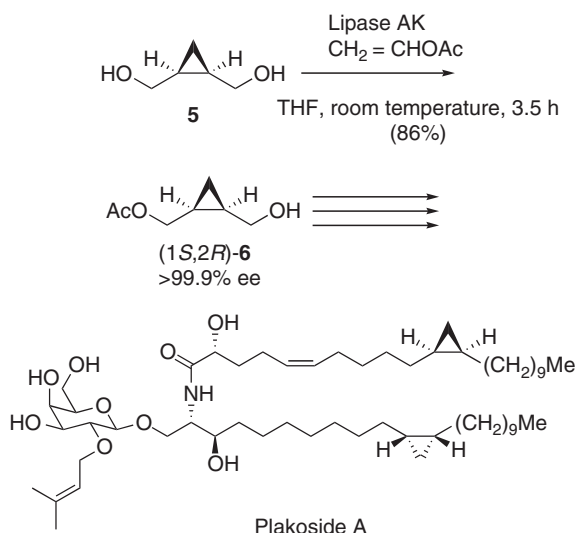


FIGURE 22.4 Preparation and utilization of half-acetate (1*S*,2*R*)-**6**.

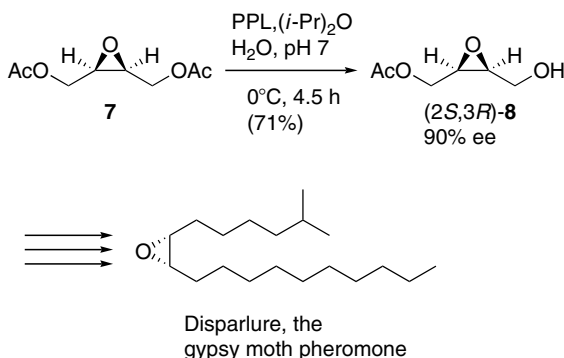


FIGURE 22.5 Preparation and utilization of half-acetate (2*S*,3*R*)-**8**.

(Figure 22.5) [9]. This half-acetate **8** could be converted to various pheromones as shown in Figure 22.6. Alkylative ring-cleavage of epoxides afforded other kinds of pheromones, too. Chuche and coworkers reported the preparation of a similar building block [10]. In 2003, it became clear that enzymatic kinetic resolution of (1*S**,2*R**)-(±)-4-*t*-butyldiphenylsilyloxy-2,3-epoxy-1-butanol gives enantiomerically pure (98 to 99% ee) and therefore more useful building block (see Section 22.4.1)

22.2.4.1 Preparation of (2*S*,3*R*)-**8** [9]

PPL (Sigma, 1.2 g) was added to a solution of **7** (2.82 g, 15 mmol) in (*i*-Pr)₂O (67 mL) and 0.1 M phosphate buffer (pH 7, 140 mL) at 0°C. The mixture was stirred vigorously at 0°C for 4.5 h, and the pH was kept constant by addition of 1 M NaOH using an autoburette. After addition of NaOH (1 equivalent), the (*i*-Pr)₂O layer was separated and the aqueous layer was extracted with Et₂O. The combined organic solution was dried (MgSO₄) and concentrated *in vacuo*. The residue was chromatographed on SiO₂. Elution with hexane/Et₂O (2:1 to 1:1) afforded **8** as a slightly yellow oil (1.55 g, 71%), $n_D^{20} = 1.4476$; $[\alpha]_D^{22} = +17.4$ ($c = 0.9$, CH₂Cl₂). Enantiomeric purity determination: derivatization followed by HPLC analysis, 90% ee.

22.2.5 (2*S*,3*R*)-4-ACETOXY-2,3-ISOPROPYLIDENEDIOXY-1-BUTANOL (**10**)

Stereochemically defined *vic*-diols can be starting materials for stereochemically defined 6,8-dioxabicyclo[3.2.1]octanes, a common structure-type among insect pheromones. As shown in Figure 22.7, erythritol derivative **9** was acetylated with vinyl acetate using lipase AK (Amano) to furnish (2*S*,3*R*)-**10** (98.5% ee) in 96% yield [11]. Vandewalle and coworkers also obtained (2*S*,3*R*)-**10** by employing lipase SAM II (Amano-Fluka) [12]. (+)-*endo*-Brevicomin, a bark beetle pheromone, can be prepared from (2*S*,3*R*)-**10** [11].

22.2.5.1 Preparation of (2*S*,3*R*)-**10** [11]

A mixture of **9** (5.60 g, 34.5 mmol) and lipase AK (Amano, 800 mg) in vinyl acetate (7.0 mL) was stirred at room temperature for 5 h. The mixture was filtered through a Celite pad, which was washed with EtOAc. The filtrate was concentrated *in vacuo*. The residue was chromatographed on SiO₂ (80 g). Elution with hexane/EtOAc (2:1) gave **10** (6.75 g, 96%) as an oil, $n_D^{21} = 1.4503$; $[\alpha]_D^{21} = +17.3$ ($c = 1.08$, CHCl₃). Enantiomeric purity determination: derivatization to the benzoate and HPLC analysis on Chiralcel OG), 98.5% ee.

22.2.6 (*R*)-2-Acetoxymethyl-3-Buten-1-ol (**12**)

To synthesize the β -lactone moiety of an antibiotic 1233A, (*R*)-**12** was designed and prepared by asymmetric hydrolysis of prochiral diacetate **11** with lipase P (Amano) to give (*R*)-**12** (90% ee) in 86% yield (Figure 22.8) [13]. Asymmetric acetylation or hydrolysis serves as a tool to convert prochiral diacetate or diol to an optically active building block.

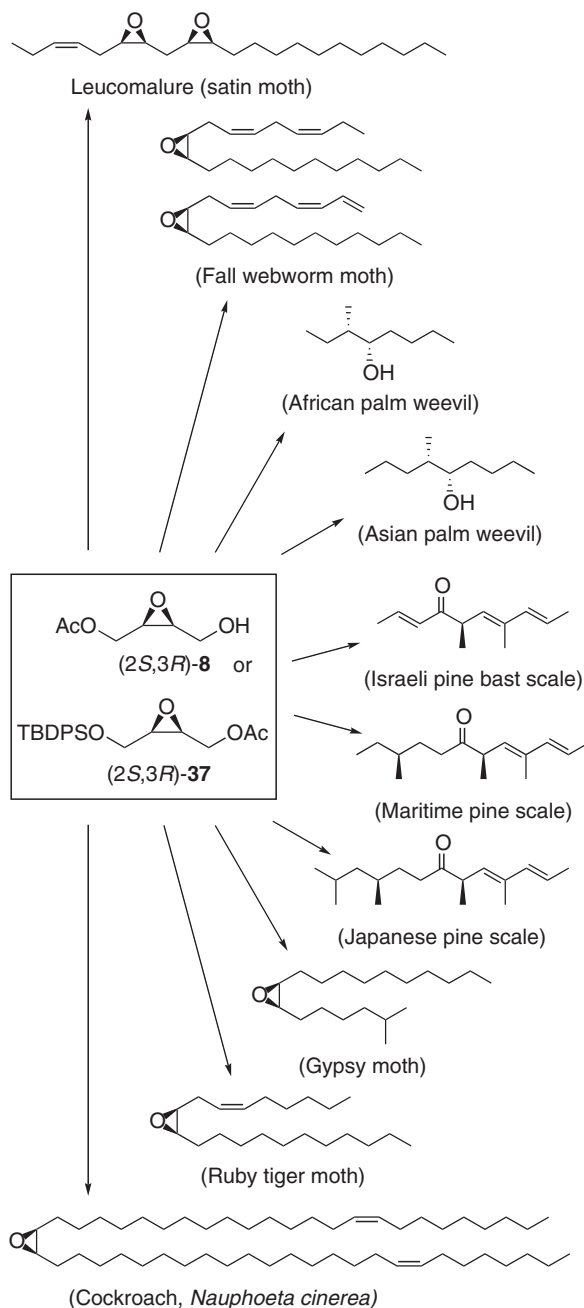


FIGURE 22.6 Pheromones synthesized from (2*S*,3*R*)-**8** and (2*S*,3*R*)-**37**.

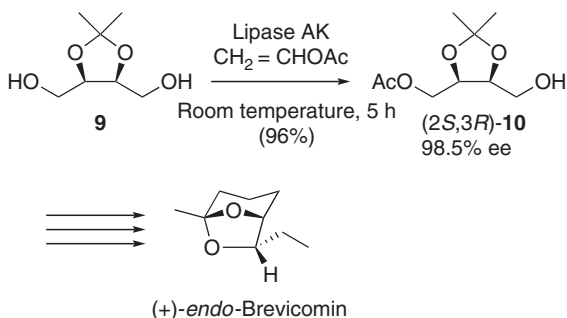


FIGURE 22.7 Preparation and utilization of half-acetate $(2S,3R)\text{-10}$

22.3 ASYMMETRIC REDUCTION OF PROCHIRAL KETONES WITH YEASTS

22.3.1 $(S)\text{-1-Phenylthio-2-Butanol}$ (**14**)

Baker's yeast (*Saccharomyces cerevisiae*) reduced 1-thiophenyl-2-butanone (**13**) to $(S)\text{-14}$ (91% ee) using ethanol as the energy source (Figure 22.9) [14]. Oxidation of **14** with *tert*-butyl hydroperoxide afforded crystalline **15**, which could be purified by recrystallization. Sulfoxide **15** served as the starting material for the synthesis of $(+)\text{-juvabione}$, the well-known juvenile hormone mimic [14].

22.3.1.1 Preparation of $(S)\text{-14}$ [14]

A suspension of dry baker's yeast (Oriental Yeast Co., 300 g) in H_2O (3 L) was stirred for 10 min. A solution of **13** (9.03 g, 50.1 mmol) in 95% EtOH (20.0 mL) was divided into ten portions and added every 30 to 150 min to the vigorously stirred and aerated yeast suspension at room temperature. When the evolution of CO_2 gas was too vigorous, a few mL of 2% aqueous antifoam (AF) emulsion or 95% EtOH was added. After 19 h, baker's yeast (83 g) and 95% EtOH (10 mL) were added, and vigorous stirring and aeration were continued for a total of 2 d. At the end of this period, the total volume of EtOH added was 200 mL. The reaction was stopped by the addition of Et_2O , and the mixture was filtered through Celite. The filtrate was saturated with NaCl, and extracted three times with EtOAc. The filter-cake was suspended in acetone, sonicated, and filtered. The filtrate was concentrated *in vacuo*. The residue was extracted three times with EtOAc. The combined extracts were washed with saturated NaHCO_3 aqueous solution and brine, dried (MgSO_4), and concentrated *in vacuo*.

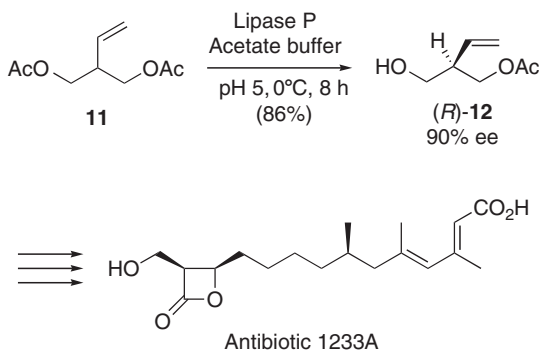


FIGURE 22.8 Preparation and utilization of half-acetate $(R)\text{-12}$.

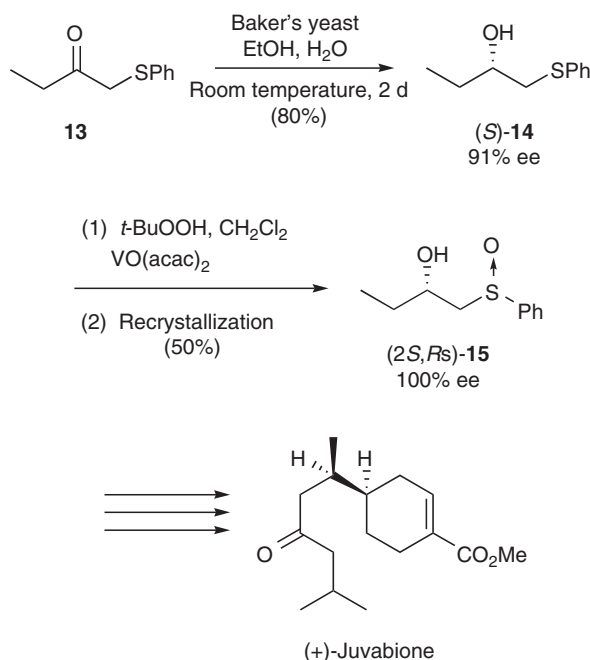


FIGURE 22.9 Preparation and utilization of (*S*)-14.

The residue (12.9 g) was chromatographed on SiO₂ (400 g, hexane/EtOAc 10:1) to give 8.50 g (80%) of (*S*)-14 as an oil, $n_D^{18} = 1.5589$; $[\alpha]_D^{22} = +52.5$ ($c = 1.66$, CHCl₃). Enantiomeric purity determination: HPLC analysis after derivatization to (*R*)-MTPA ester, 91% ee. After oxidation of (*S*)-14 to (*2S, Rs*)-15, its enantiomeric purity was improved to ~100% ee by recrystallization.

22.3.2 (*S*)-3-HYDROXY-2,2-DIMETHYLCYCLOHEXANONE (17)

Reduction of 2,2-dimethylcyclohexane-1,3-dione (**16**) with baker's yeast in a sucrose solution gave (*S*)-17 (99% ee) in 70 to 80% yield (Figure 22.10) [15,16]. Many terpenoids as shown in Figure 22.11 were synthesized from (*S*)-17, including glycinoeclepin A [17] and insect juvenile hormone III [18]. This hydroxy ketone (*S*)-17 is one of the most useful chiral building blocks of microbial origin, and affords not only cyclic terpenoids but also acyclic terpenoids like juvenile hormone III after Baeyer–Villiger oxidation.

22.3.2.1 Preparation of (*S*)-17 [15,16]

A solution of **16** (15.0 g, 107 mmol) in 95% EtOH (30 mL) and 0.2% Triton X-100 solution (150 mL) was added to a suspension of dry baker's yeast (Oriental Yeast Co., 200 g) in a 15%

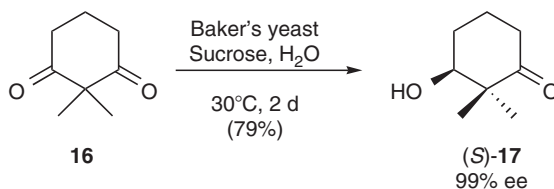


FIGURE 22.10 Preparation of hydroxy ketone (*S*)-17.

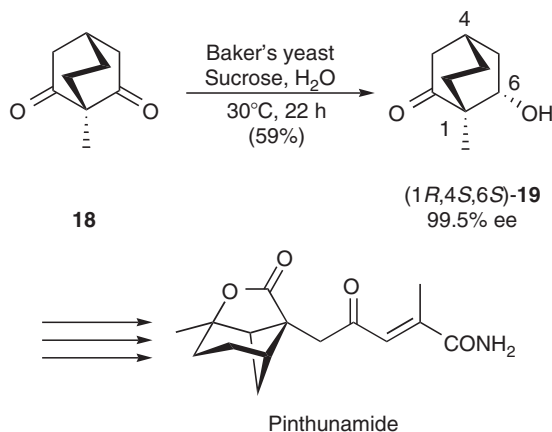


FIGURE 22.12 Preparation and utilization of hydroxy ketone (1*R*,4*S*,6*S*)-**19**.

elution with EtOAc gave 8.8 g (79% based on the consumed **16**) of (*S*)-**17**, bp 85 to 87°C/83.7 Torr; $n_D^{21} = 1.4747$; $[\alpha]_D^{21} = +24.1$ ($c = 1.12$, CHCl_3). Enantiomeric purity determination: HPLC analysis after derivatization to (*S*)-MTPA ester, 99% ee.

22.3.3 (1*R*,4*S*,6*S*)-6-HYDROXY-1-METHYLBICYCLO[2.2.2]OCTAN-2-ONE (**19**)

Reduction of bridged bicyclic ketone **18** with baker's yeast in a sucrose solution furnished crystalline (1*R*,4*S*,6*S*)-**19** (99.5% ee) in 59% yield (Figure 22.12) [19]. This hydroxy ketone **19** served as the starting material for the synthesis of pinthunamide [20] and other sesquiterpenes.

22.3.3.1 Preparation of (1*R*,4*S*,6*S*)-**19** [19]

A solution of **18** (0.79 g, 5.2 mmol) in 99% EtOH (6 mL) was reduced with baker's yeast (7 g) and sucrose (16.8 g) in H_2O (80 mL) in the presence of Triton S-100 (0.2% aqueous solution, 6 mL) at 30°C for 22 h. Subsequent work-up followed by SiO_2 chromatography as described for (*S*)-**17** gave 0.6 g of crude but crystalline **19**. This was recrystallized from hexane/EtOAc 85:1 to give **19** (0.47 g, 59%) as needles, mp 117.5 to 118.0°C; $[\alpha]_D^{16} = -5.6$ ($c = 1.0$, CHCl_3), 98.8% de [as determined by gas chromatography (GC)]. Determination of enantiomeric purity: HPLC analysis after derivatization to MTPA ester, 99.5% ee.

22.3.4 OTHER 3-HYDROXY KETONES

Figure 22.13 illustrates the preparation of some other 3-hydroxy ketones. Reduction of **20** was not stereoselective with baker's yeast. However, by employing another yeast, *Pichia terricola* KI 0117, **20** was stereoselectively reduced to (2*S*,3*S*)-**21** (99% ee), which was converted to the naturally occurring (+)-juvenile hormone I [21]. 1,3-Diketone **22** was reduced with baker's yeast to give (1*R*,4*S*,6*S*)-**23** [19], which was converted to (+)-juvabione, a juvenile hormone mimic [22].

4-Methylbicyclo[2.2.1]heptane-2,6-dione (**24**) was unstable in H_2O even at pH 7 due to the ring strain, and its reduction was only successful by employing a large amount of baker's yeast in the presence of phosphate buffer (pH 7) to give (1*R*,4*S*,6*S*)-6-hydroxy-4-methylbicyclo[2.2.1]heptan-2-one (**25**, 82.5% ee) in 42% yield, [17]. The right-hand portion of glycinoeclepin A was constructed from **25** [17].

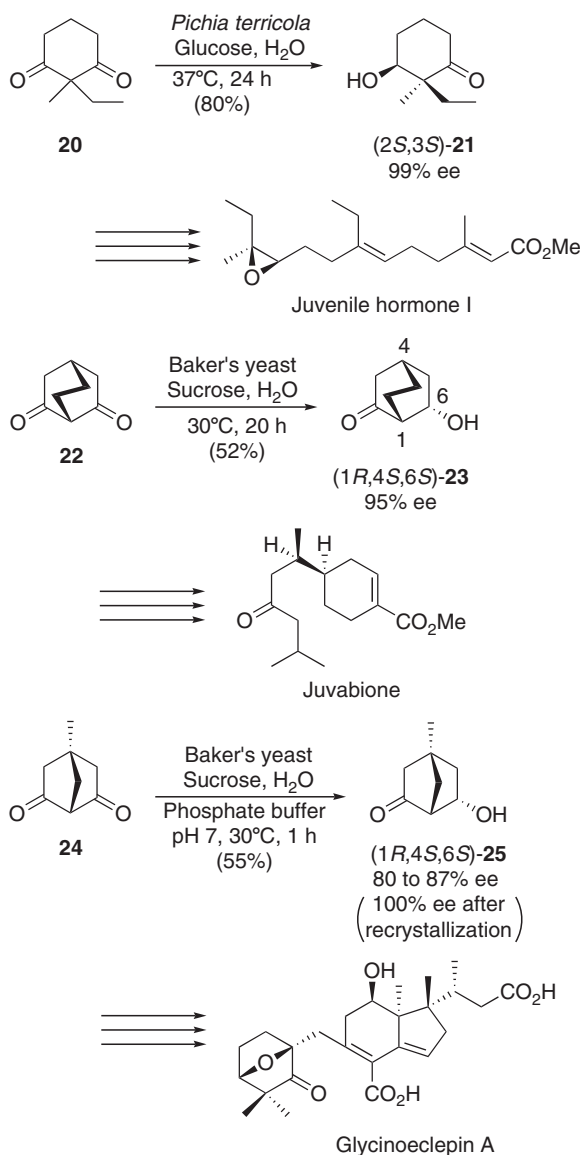


FIGURE 22.13 Preparation and utilization of hydroxy ketone (2*S*,3*S*)-**21**, (1*R*,4*S*,6*S*)-**23**, and (1*R*,4*S*,6*S*)-**25**.

22.3.4.1 Preparation of (1*R*,4*S*,6*S*)-**25** [17]

A suspension of dry baker's yeast (Oriental Yeast Co., 100 g), sucrose (100 g), KH_2PO_4 (5.5 g), and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (21.5 g) in H_2O (1 L) was kept at 30°C on a rotary platform shaker for 10 min. A solution of **24** (1.0 g, 7.2 mmol) in 95% EtOH (3 mL) was then added to the mixture, and the incubation was continued at 30°C. After 10 min, sucrose (10 g) and dry baker's yeast (10 g) were again added to the mixture, and after a further 2 min of incubation, dione **24** (1.0 g, 7.2 mmol) was also added to the mixture. These additions of sucrose, baker's yeast, and **24** were repeated twice more. After the completion of the additions, the incubation was continued for 30 min. The mixture was then filtered through Celite, and the filter-cake was washed with acetone. The filtrate was made slightly alkaline (pH ~8, universal indicator)

by addition of NaHCO_3 , and was then saturated with NaCl and extracted six times with EtOAc . The washings (acetone solution) were evaporated, and the residue was diluted with EtOAc and washed with saturated aqueous NaHCO_3 . The combined organic phase was dried (MgSO_4), filtered, and concentrated *in vacuo*. The residue was purified by SiO_2 chromatography to give **25** as crystals (2.04 g, 55%), mp 56.5 to 57.5°C (prisms from hexane/ Et_2O); $[\alpha]_{\text{D}}^{23} = -16.6$ ($c = 1.06$, CHCl_3). Enantiomeric purity determination: HPLC analysis after derivatization to MTPA ester, 82.5% ee.

22.3.5 OCTAHYDRONAPHTHALENONE (28) AND OCTAHYDROINDENONE (31)

Sugai and coworkers selected a yeast strain, *Torulaspora delbrueckii* IFO 10921, for the selective reduction of a carbonyl group of triketones **26** and **29** (Figure 22.14) [23]. Structure of the reduction product of **26** was determined as (1*S*,3*S*,6*S*)-**27** by x-ray analysis. The hemiacetal ketone **27** was converted to (1*S*,8*aR*)-**28**, a useful building block in isoprenoid synthesis [23]. Reduction of **29** gave **30**, which was not analyzed by x-ray [24]. The hemiacetal ketone **30** yielded (1*S*,7*aR*)-**31**, another useful building block in steroid synthesis [24].

22.3.6 CYCLIC AND ENOLIZABLE β -KETO ESTERS WITH A SINGLE STEREOGENIC CENTER AT α -POSITION

Yeast reduction of a cyclic and enolizable β -keto ester with a single stereogenic center at α -position is known to give a single optically active β -hydroxy ester in more than 50% yield, because enolization of the starting β -keto ester destroys the chirality at the α -position before

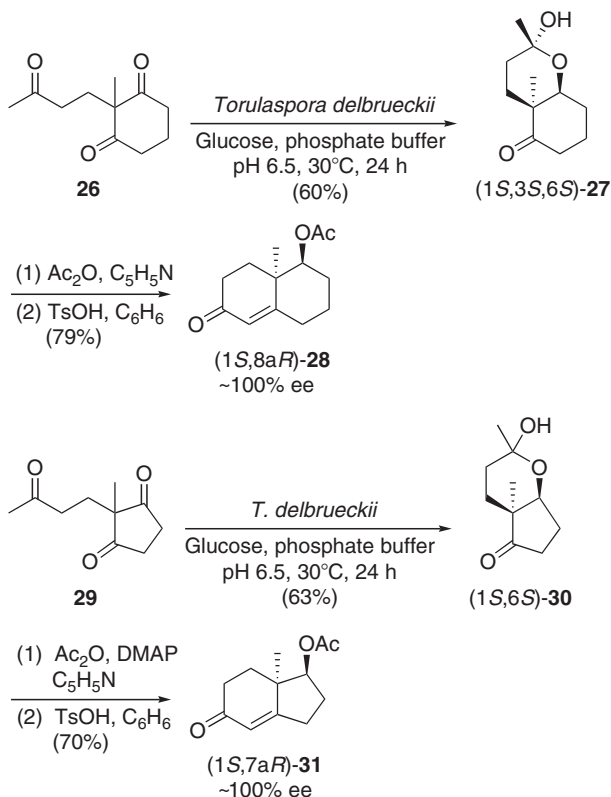


FIGURE 22.14 Preparation of octahydronaphthalenone **28** and octahydroindenone **31**.

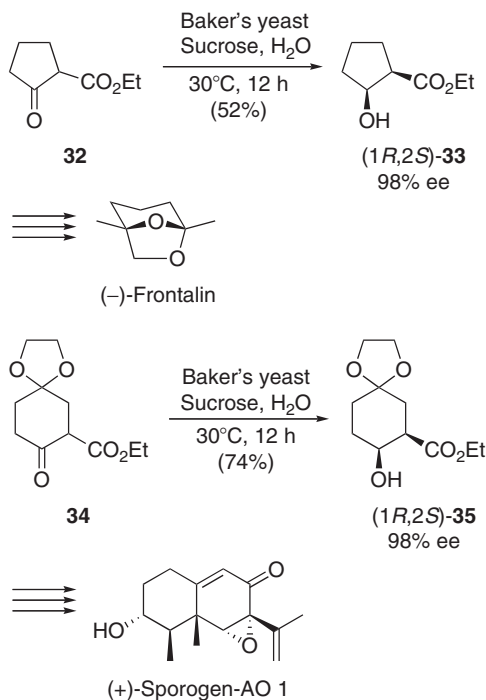


FIGURE 22.15 Preparation and utilization of cyclic β -hydroxy esters (1*R*,2*S*)-**33** and (1*R*,2*S*)-**35**.

reduction. As shown in Figure 22.15, reduction of **32** with baker's yeast gave ethyl (1*R*,2*S*)-2-hydroxycyclopentane-1-carboxylate (**33**, 98% ee) in 53% yield, and **33** was converted to (–)-frontalin, a bark beetle pheromone [25].

Reduction of **34** with baker's yeast is also an efficient method to obtain a useful building block, ethyl (1*R*,2*S*)-5,5-ethylenedioxy-2-hydroxycyclohexane-1-carboxylate (**35**, 98% ee) in 74% yield [26], which eventually afforded (+)-sporogen-AO 1, a microbial bioregulator [27].

22.3.6.1 Preparation of (1*R*,2*S*)-**33** [25]

A solution of **32** (27.2 g, 174 mmol) in 95% EtOH (10 mL) was added dropwise with stirring to a suspension of dry baker's yeast (Oriental Yeast Co., 140 g) in a solution of sucrose (300 g) in H₂O (2.5 L). The mixture was stirred and aerated at 30°C for 12 h. Occasionally *n*-C₈H₁₇OH (a few drops) was added to the suspension to suppress the foaming. The mixture was filtered through Celite, and the filtration bed was washed with EtOAc. The combined filtrate and washings were washed with brine, dried (MgSO₄), and concentrated *in vacuo*. The residue was distilled to give crude **33**. This was chromatographed on SiO₂ (300 g, hexane/EtOAc = 50:1). The fractions containing **33** were further purified by distillation to give 14.4 g (52%) of **33**, bp 76°C/4 Torr; $n_D^{25} = 1.4534$; $[\alpha]_D^{22} = +14.5$ ($c = 2.83$, CHCl₃). Enantiomeric purity determination: GC on Chirasil DEX-CB, 97.8% ee.

22.3.6.2 Preparation of (1*R*,2*S*)-**35** [26,27]

A suspension of dry baker's yeast (Oriental Yeast Co., 200 g) in a solution of sucrose (300 g) in H₂O (2 L) was stirred for 15 min at 30°C with aeration. A solution of **34** (15 g, 66 mmol) in 95% EtOH (30 mL) was added to this, and the fermentation was continued overnight at 30°C

with stirring and aeration. Then NaHCO_3 (50 g) and EtOAc (500 mL) were added, and the mixture was filtered through Celite. The filtrate was saturated with NaCl , and extracted with EtOAc five times. The filter-cake was washed thoroughly with EtOAc . The combined EtOAc solution was washed with brine, dried (MgSO_4), and concentrated *in vacuo*. The residue was chromatographed on SiO_2 (400 g, hexane/ EtOAc 4:1 to 1:1) to give 1 to 2 g of recovered **34** and 9 to 10 g (60 to 67%) of **35**, bp 117 to 118°C/0.35 Torr; $n_D^{25} = 1.4695$; $[\alpha]_D^{23} = +51.1$ ($c = 1.02$, CHCl_3). Enantiomeric purity determination: HPLC analysis of the corresponding MTPA ester, 98.4% ee.

22.4 KINETIC RESOLUTION OF RACEMIC ALCOHOLS, HYDROXY ACIDS, AND AMINO ACIDS WITH HYDROLYTIC ENZYMES

22.4.1 (2*R*,3*R*)-1-Acetoxy-4-(*t*-BUTYLDIPHENYLSILOXY)-2,3-EPOXYBUTANE (**37**)

Asymmetric acetylation of (\pm)-epoxy alcohol **36** with lipase PS-C (Amano) and vinyl acetate gave acetate (2*S*,3*R*)-**37** (98.1% ee) and the unreacted alcohol (2*R*,3*S*)-**36** (99.5% ee) in 48 and 49% yields, respectively (Figure 22.16) [28]. These products could be obtained with satisfactory enantiomeric purities through a single enzymatic reaction. This asymmetric acetylation procedure is therefore more efficient than the asymmetric hydrolysis of *meso*-epoxy diacetate **7** (see Section 22.2.4) with regard to the optical yield (98 to 99% ee vs. 90% ee). The epoxide (2*S*,3*R*)-**37** was converted to (3*Z*,6*R*,7*S*,9*R*,10*S*)-leucomalure, the sex pheromone of the satin moth [28].

22.4.1.1 Preparation of (2*S*,3*R*)-**37** and (2*R*,3*S*)-**36** [28]

Lipase PS-C (Amano, 50 mg) was added to a solution of (\pm)-**36** (2.00 g, 5.84 mmol) in Et_2O (20 mL) and vinyl acetate (1.0 mL) at room temperature. After stirring for 5 h at room temperature, the enzyme was filtered off and the filtrate was concentrated *in vacuo*. The residue was purified by SiO_2 chromatography (hexane/ EtOAc , 5:1) to give less polar (2*S*,3*R*)-**37** (1.07 g, 48%), $n_D^{22} = 1.5359$; $[\alpha]_D^{22} = -3.66$ ($c = 1.25$, CH_2Cl_2), and more polar (2*R*,3*S*)-**36** (971 mg, 49%), $n_D^{22} = 1.5161$; $[\alpha]_D^{23} = +6.5$ ($c = 1.0$, CH_2Cl_2). Enantiomeric purity determination of **36**: HPLC analysis on Chiralcel OD, 99.5% ee for (2*R*,3*S*)-isomer and 98.1% ee for (2*S*,3*R*)-isomer.

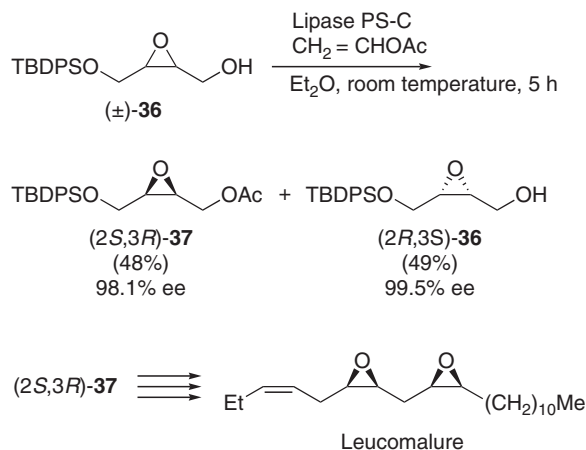


FIGURE 22.16 Preparation and utilization of epoxide (2*S*,3*R*)-**37**.

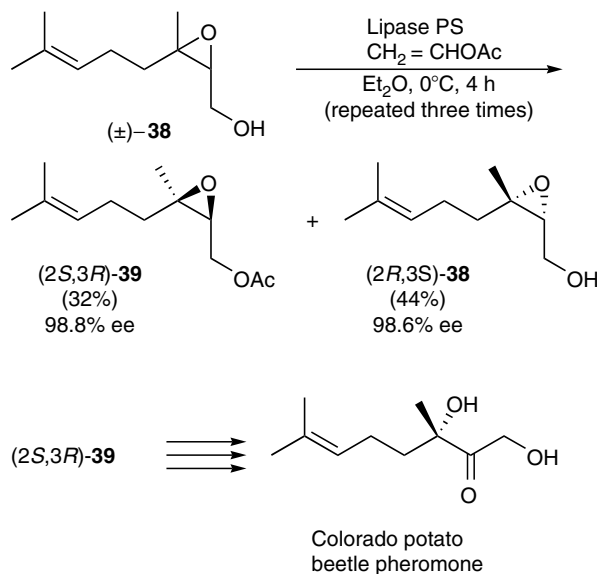


FIGURE 22.17 Preparation and utilization of epoxy acetate (2*S*,3*R*)-39.

22.4.2 (2*S*,3*R*)-3,7-DIMETHYL-2,3-EPOXY-6-OCTENYL ACETATE (39) AND (2*R*,3*S*)-3,7-DIMETHYL- 2,3-EPOXY-6-OCTEN-1-OL (38)

Asymmetric acetylation of (\pm)-2,3-epoxyneryl (**38**) with vinyl acetate using lipase PS (Amano) was repeated three times to give (2*S*,3*R*)-**39** (98.8% ee) in 32% yield and also (2*R*,3*S*)-**38** (98.6% ee) in 44% yield (Figure 22.17) [29]. The former acetate (2*S*,3*R*)-**39** was converted to the pheromone of the Colorado potato beetle [29]. Since a similar asymmetric acetylation is observed with (\pm)-2,3-epoxygeraniol to give (1*R*,3*R*)-acetate, lipase PS recognizes only the chirality at C-3 of **38** and 2,3-epoxygeraniol [29].

22.4.2.1 Preparation of (2*S*,3*R*)-39 and (2*R*,3*S*)-38 [29]

Lipase PS (Amano, 4.6 g) was added to a stirred solution of (\pm)-**38** (23.6 g, 162 mmol) and vinyl acetate (60 mL) in Et₂O (230 mL) at 0°C. The mixture was stirred at 0°C for 4 h. It was then filtered through Celite and the filtrate was concentrated *in vacuo*. The residue was chromatographed on SiO₂ (1.1 kg, hexane/EtOAc = 5:1 to 2:1) to give (2*S*,3*R*)-**39** (16.8 g, 79 mmol) and (2*R*,3*S*)-**38** (14.1 g, 83 mmol). The above (2*S*,3*R*)-**39** (16.8 g, 79 mmol) was then methanolized with K₂CO₃ (1.1 g) in MeOH (200 mL) at room temperature for 4 h. This procedure—enzymatic resolution followed by methanolysis—was repeated with the resulting (2*S*,3*R*)-**38** twice more to give (2*S*,3*R*)-**39** (5.49 g, 98.8% ee) in 32% yield, $n_D^{20} = 1.4570$, $[\alpha]_D^{23} = -25.7$ ($c = 0.58$, CHCl₃), and also (2*R*,3*S*)-**38** (5.19 g, 98.6% ee) in 44% yield, $n_D^{28} = 1.4702$; $[\alpha]_D^{28} = +13.3$ ($c = 1.09$, CHCl₃). Enantiomeric purity determination: HPLC analysis on Chiralcel OD.

22.4.3 ENANTIOMERS OF 2-(2',2'-DIMETHYL-6'-METHYLENOCYCLOHEXYL)ETHANOL (40)

(\pm)- γ -Cyclohomogeraniol (**40**, Figure 22.18) was resolved by asymmetric acetylation with vinyl acetate using lipase AK (Amano) to give (*R*)-**41** and (*S*)-**40** [30]. Lipase AK recognized the stereochemistry at C-1' of the ring-system. Ancistrodial, a defense sesquiterpene of a termite, was prepared from (*R*)-**41** [31], while (*S*)-**40** was converted to γ -coronal, an ambergris odorant [30].

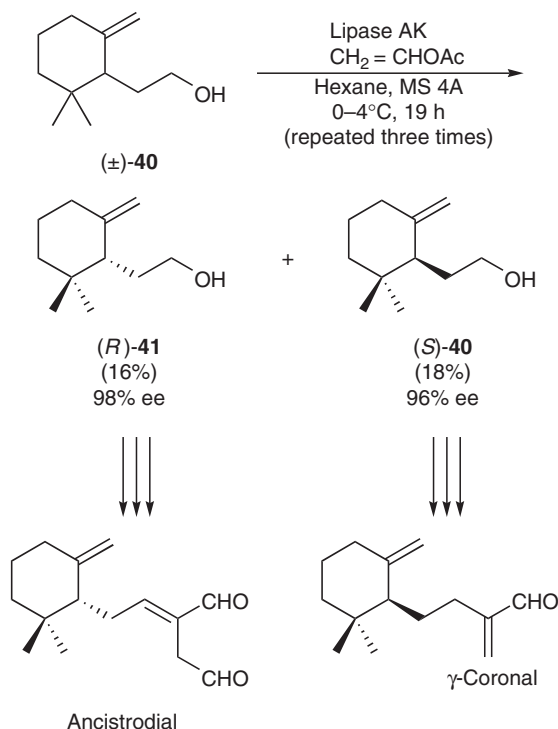


FIGURE 22.18 Preparation and utilization of the enantiomers of γ -cyclohomogeraniol **40**.

22.4.4 ENANTIOMERS OF 2,4,4-TRIMETHYL-2-CYCLOHEXEN-1-OL (**43**)

Asymmetric hydrolysis of (\pm) -2,4,4-trimethyl-2-cyclohexenyl acetate (**42**) with pig liver esterase (PLE) in phosphate buffer (pH 7.5) yielded (R) -2,4,4-trimethyl-2-cyclohexen-1-ol (**43**, ~100% ee) in 26% yield together with 67% of the recovered (S) -**42** (41% ee) (Figure 22.19) [32].

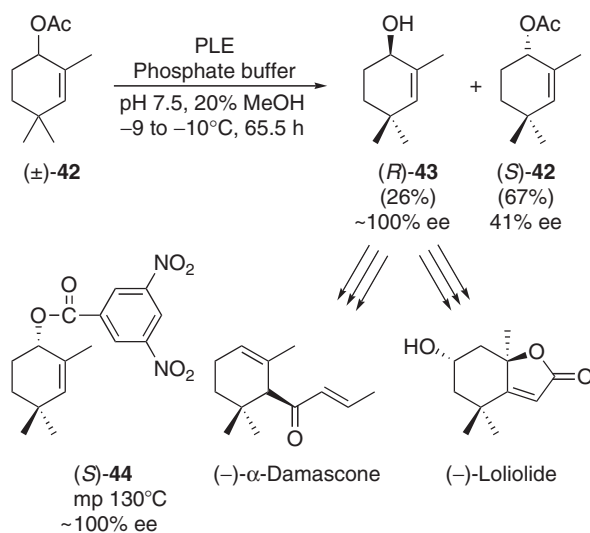


FIGURE 22.19 Preparation and utilization of the enantiomers of **43**.

The acetate (*S*)-**42** gives enantiomerically pure (*S*)-**42** after the second hydrolysis with PLE and purification with crystalline (*S*)-**44** [32]. The enantiomers of **43** proved to be extremely useful building blocks for the synthesis of terpenes and degraded carotenoids such as (–)- α -damascone and (–)-loliolide [33,34].

22.4.4.1 Preparation of (*R*)-**43** and (*S*)-**42** [32]

PLE (150 mg protein, 50,250 units) was added to a stirred mixture of (\pm)-**42** (26.3 g, 145 mmol) in 0.1 M phosphate buffer containing 20% of MeOH (1.11 L) at –9 to –10°C. The mixture was vigorously stirred for 65.5 h, keeping the reaction temperature at –9 to –10°C. Then the mixture was saturated with NaCl and (NH₄)₂SO₄, and extracted several times with Et₂O. The extract was washed with saturated NaHCO₃ solution and brine, dried (MgSO₄), and concentrated *in vacuo*. The residue (27.5 g) was chromatographed on SiO₂ (550 g, hexane/Et₂O 30:1 to 10:1) to give 17.7 g (67%) of (*S*)-**42**, bp 57 to 57.5°C/2.5 Torr; $n_D^{22} = 1.4561$; $[\alpha]_D^{21} = -39.5$ ($c = 1.10$, MeOH), 41% ee. Further elution gave 5.32 g (26%) of (*R*)-**43**, bp 63 to 64°C/3 Torr; $n_D^{22} = 1.4751$; $[\alpha]_D^{21} = +95.7$ ($c = 1.13$, MeOH), ~100% ee. Enantiomeric purity determination: derivatization to MTPA ester and HPLC analysis. The acetate (*S*)-**42** was treated with PLE once more to give (*S*)-**42** of 95.8% ee in 67% yield. Further purification could be achieved by recrystallization of (*S*)-**44**, mp 130 to 131.5°C, $[\alpha]_D^{24} = -118.5$ ($c = 1.04$, CHCl₃), ~100% ee, in 77% recovery.

22.4.5 (3*aR*,5*S*,8*bS*)-5-HYDROXY-8,8-DIMETHYL-3,3*a*,4,5,6,7,8,8*b*-OCTAHYDROINDENO[1.2-*b*]FURAN-2-ONE (**45**)

Our synthesis of (+)-strigol, a potent stimulant for the germination of parasitic weeds, necessitated an efficient synthesis of (+)-**45** (Figure 22.20). Asymmetric acetylation of (\pm)-**45** afforded (+)-**45** (99% ee) and (+)-**46** (87% ee) [35]. The acetate (+)-**46** could be further purified by recrystallization. This is a very simple method to prepare (+)-**45**, which was converted to (+)-strigol [35].

22.4.6 (*R*)-3-HYDROXY-15-METHYLHEXADECANOIC ACID (**47**) AND (*R*)-2-HYDROXY-21-METHYLDOCOSANOIC ACID (**48**)

These long-chain α - or β -hydroxy acids **47** and **48** are known as the acyl side-chain portions of sphingolipids. The natural hydroxy acids are (*R*)-isomers and are highly crystalline. It is therefore possible to prepare (*R*)-acids by treating (\pm)-acids with lipase PS and vinyl acetate, and collecting the crystalline (*R*)-acids that have remained intact after the acylation. Accordingly, (\pm)-**47** gave (*R*)-3-hydroxy-15-methylhexadecanoic acid (**47**, 100% ee) in 28% yield (Figure 22.21) [36]. Due to the low reactivity of **47**, the reaction was carried out at a higher temperature (60°C), and BHT (2,6-di-*t*-butyl-4-methylphenol, a polymerization inhibitor) was added to the reaction mixture to prevent polymerization of vinyl acetate. (–)-Sulfobacin A, an inhibitor of DNA polymerase α , was synthesized employing (*R*)-**47** [36]. Similarly, (\pm)-**48** furnished (*R*)-2-hydroxy-21-methyldocosanoic acid (**48**, 98% ee) in 25% yield, which was used for the synthesis of the sex pheromone of the female hair crab [37].

22.4.6.1 Preparation of (*R*)-**47** [36]

Lipase PS (Amano, 1.00 g) was added to a stirred solution of (\pm)-**47** (2.00 g, 6.98 mmol) and 2,6-di-*t*-butyl-4-methylphenol (BHT, 20 mg, a polymerization inhibitor) in vinyl acetate (30 mL), and the mixture was stirred at 60°C for 48 h. After cooling, the mixture was filtered and the filtrate was concentrated *in vacuo*. The residue was chromatographed on SiO₂, and

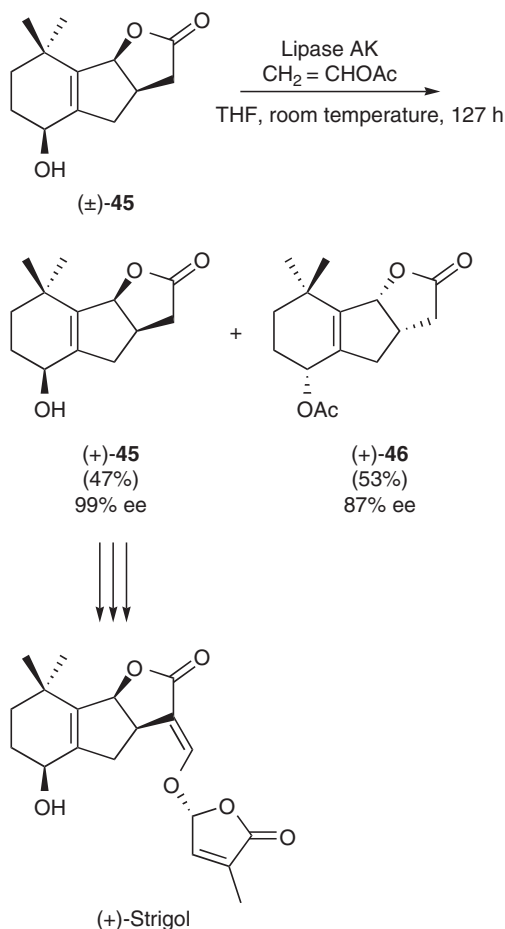


FIGURE 22.20 Preparation and utilization of hydroxy lactone (+)-45.

the resulting solid was recrystallized as colorless plates of (*R*)-47 (562 mg, 28%, mp 55 to 57°C [α]_D²³ = −12.7 (*c* = 1.02, CHCl₃). Enantiomeric purity determination: gas–liquid chromatography (GLC) analysis of the corresponding methyl ester on Chirasil-DEXCB, ~100% ee.

22.4.7 (2*R*,3*S*)- AND (2*S*,3*R*)-3-ACETOXY-2-(OCTADECANOYLAMINO)-1-HEXADECANOL (50)

Sphingolipids are important biofunctional molecules. Sugai and coworkers developed an enzymatic method to prepare both the natural (2*S*,3*R*)- and unnatural (2*R*,3*S*)-isomers of 3-acetylated ceramide **50** (Figure 22.22) [38]. By employing an immobilized form of SC lipase A from *Burkholderia cepacia* (Sumitomo Chemical Co.), (±)-49 was regio- and enantioselectively hydrolyzed to give (2*R*,3*S*)-50 (41% yield, 98% ee) belonging to the unnatural stereochemical series. The unreacted (2*S*,3*R*)-diacetate **49** (58% yield, 69% ee) could be further purified by repeating once more the enzymatic hydrolysis to give (2*S*,3*R*)-49 of 96% ee. Curiously, when nonimmobilized SC lipase A was employed, (2*S*,3*R*)-49 (96% ee) could be hydrolyzed to give (2*S*,3*R*)-50 (~100% ee) in 85% yield. Accordingly, both the enantiomers of **50** became available. Due to the total insolubility of **49** in aqueous solvents, a biphasic system (decane/0.1 M phosphate buffer) was used as the reaction medium of enzymatic hydrolysis [38].

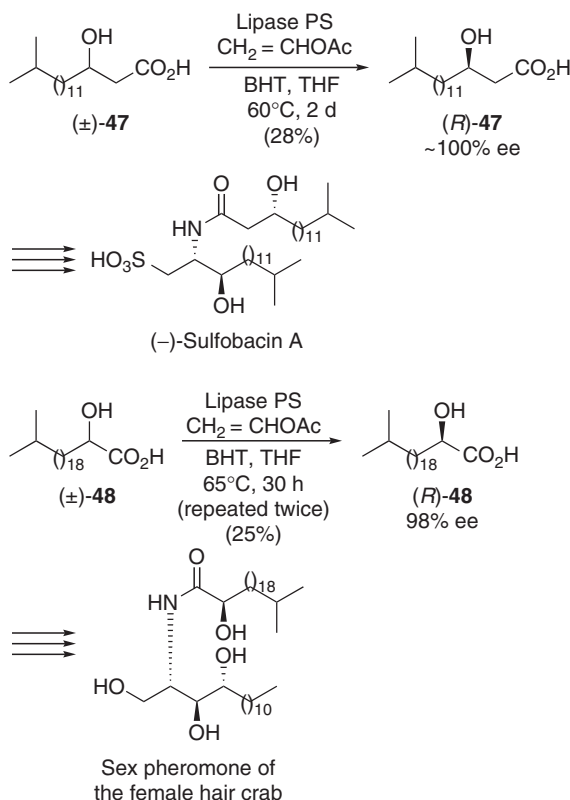


FIGURE 22.21 Preparation and utilization of hydroxy acids (R)-47 and (R)-48.

22.4.8 (4*S*,6*S*)-4-HYDROXY-4,9-DIOXASPIRO[5.5]UNDECANE (52) AND (R)-TETRAHYDROPYRANYL ALCOHOL 53

Heterocyclic alcohols can also be substrates for esterases and lipases. Acetate (±)-51 with a spiroacetal ring system was asymmetrically hydrolyzed to give alcohol (4*S*,6*S*)-52 (99% ee) and the unreacted acetate (4*R*,6*R*)-51 (~100% ee) in 22 and 13% yield, respectively, after repeating the enzymatic hydrolysis for three times (Figure 22.23) [39]. The alcohol (4*S*,6*S*)-52 is a component of the sex pheromone of the olive fruit fly [39].

Asymmetric acetylation of tetrahydropyranyl alcohol (±)-53 afforded (*S*)-acetate 54 and unreacted (*R*)-alcohol 53, the latter of which was oxidized to (*R*)-(+)-hippospongiic acid A, a metabolite of the marine sponge *Hippospongia* sp. (Figure 22.23) [40].

22.4.9 (R)- AND (S)-2-AMINOHEXADECANOIC ACID (56)

Amino acylase of *Aspergillus* origin is known to possess broad substrate specificity. *N*-Chloroacetyl derivative 55 of (±)-2-aminohexadecanoic acid (56) was treated with the acylase to give (*S*)-56 and (*R*)-55 in good yield (Figure 22.24) [41]. Removal of the *N*-chloroacetyl group of (*R*)-55 afforded (*R*)-56, which was treated with nitrous acid to furnish (*R*)-2-hydroxyhexadecanoic acid. This α-hydroxy acid is a useful building block in sphingolipid synthesis [41].

22.4.9.1 Preparation of the Enantiomers of 56 [41]

Amino acylase (from *Aspergillus*, 10,000 unit/g, Tokyo Kasei Co., 5 g) and CoCl₂ (10 mg) were added to a solution of (±)-55 (36.0 g, 103 mmol) in H₂O (4 L) adjusted to pH 7.3 by the

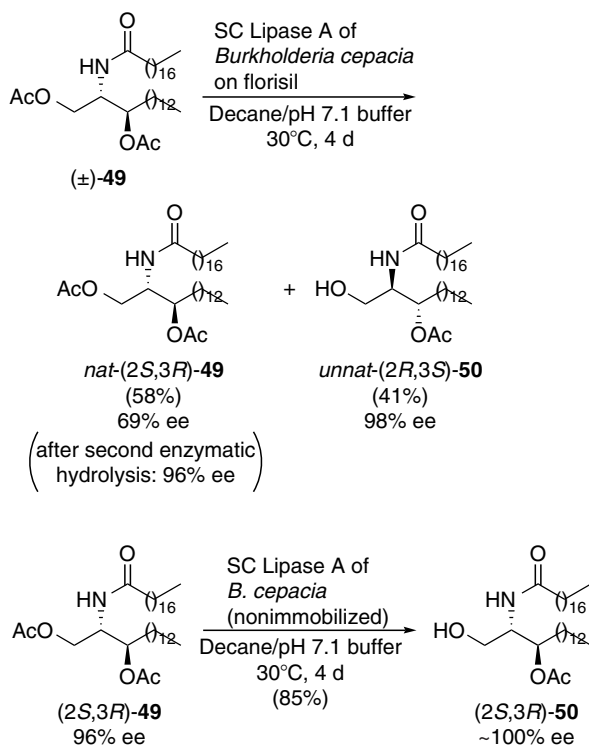


FIGURE 22.22 Preparation of natural and unnatural ceramides **50**.

addition of NaOH. The solution was left to stand for 44 h at 37°C. The precipitated crystalline (*S*)-**56** was collected on a filter, washed with MeOH and Et₂O, and dried over P₂O₅ to give 14.0 g (quantitative) of (*S*)-**56**, mp 234 to 236°C, $[\alpha]_D^{26} = +21.8$ ($c = 0.1$, AcOH). The filtrate obtained after removal of (*S*)-**56** was acidified with 3 M HCl. The precipitated solid was collected on a filter, and dissolved in EtOAc (1 L). The insoluble material was filtered off and the filtrate was concentrated *in vacuo*. The residue was recrystallized from hexane to give (*R*)-**55** (15.5 g, 86%), mp 87 to 88°C, $[\alpha]_D^{21} = -28.0$ ($c = 0.5$, CHCl₃). This acid (*R*)-**55** (15.5 g) was mixed with 4 M HCl (150 mL), and the mixture was stirred and heated under reflux for 3 h. After cooling, the mixture was neutralized with 28% NH₃ aqueous solution. The precipitated solid was collected on a filter, washed with H₂O, MeOH, and Et₂O, and dried over P₂O₅ to give (*R*)-**56** (12.0 g, 99.5%), mp 233 to 236°C, $[\alpha]_D^{26} = -21.0$ ($c = 0.1$, AcOH).

22.5 KINETIC RESOLUTION OF RACEMIC KETONES BY ASYMMETRIC REDUCTION WITH YEASTS

22.5.1 (1*S*,2*R*,5*R*)-2-ETHOXYCARBONYL-7,7-ETHYLENEDIOXYBICYCLO [3.3.0]OCTAN-3-ONE (**57**) AND (1*S*,5*R*,6*R*,7*S*)-6-ETHOXYCARBONYL-3,3-ETHYLENEDIOXY-7-HYDROXYBICYCLO[3.3.0]OCTANE (**58**)

β-Keto ester (1*S*,2*R*,5*R*)-**57** was required as the starting material for the synthesis of (+)-carbaprostaglandin I₂ (Figure 22.25). Reduction of (±)-**57** with *S. bailli* KI 0116 afforded (1*S*,2*R*,5*R*)-**57** (93.6% ee) and (1*S*,5*R*,6*R*,7*S*)-**58** (99.2% ee) in 40 and 36% yield, respectively [42]. When baker's yeast was employed, the resulting (1*S*,2*R*,5*R*)-**57** was only of 62% ee [42]. It is important to select an appropriate microorganism for the desired reaction.

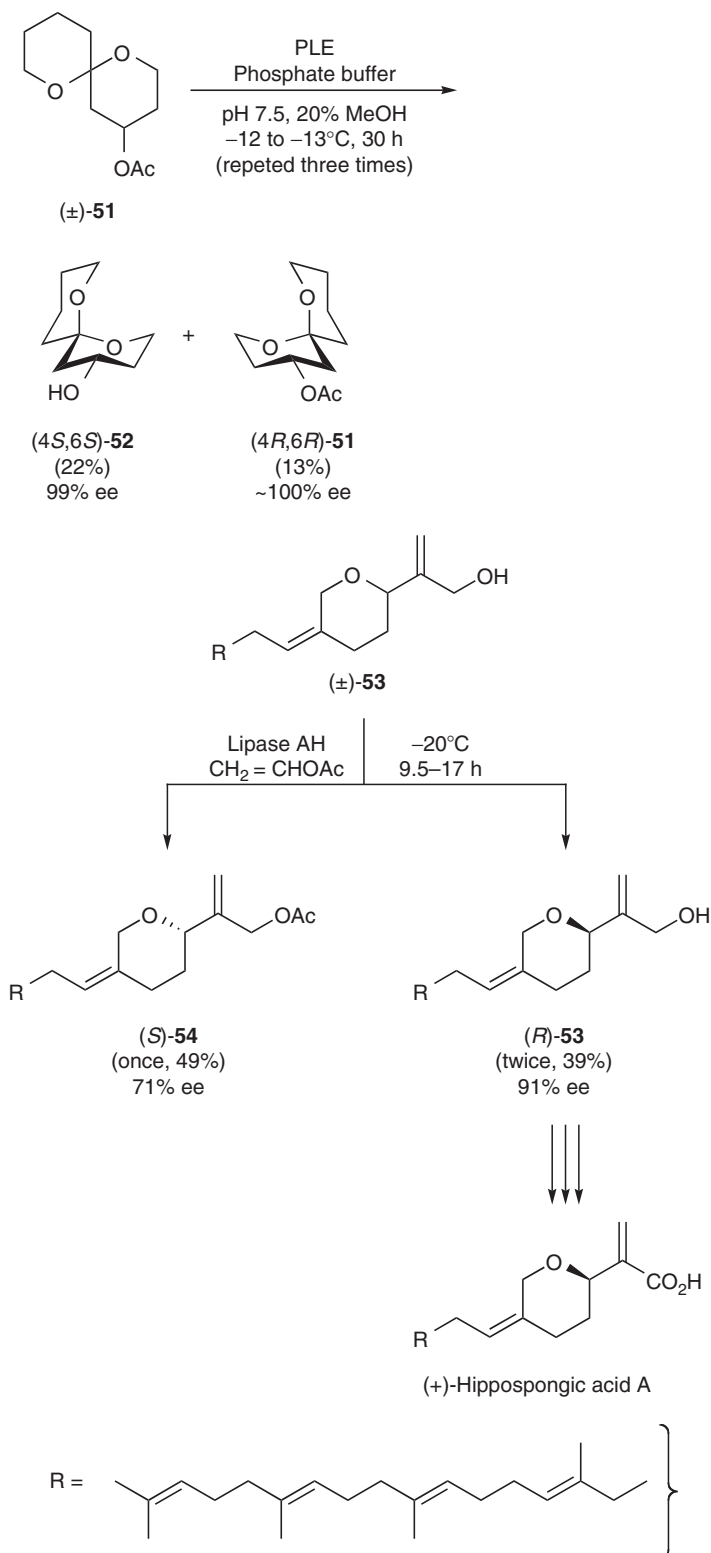


FIGURE 22.23 Preparation of oxygen heterocycles $(4S,6S)\text{-52}$ and $(R)\text{-53}$.

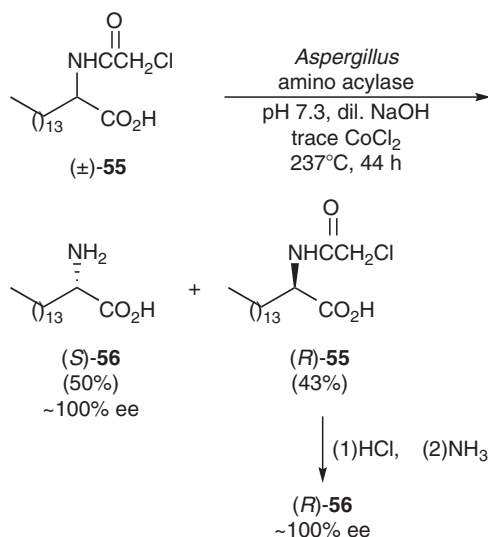


FIGURE 22.24 Preparation of the enantiomers of amino acid **56**.

22.5.2 (R)- AND (S)-WIELAND–MIESCHER KETONE (**59**)

Wieland–Miescher ketone (**59**, Figure 22.26) is a well-known and versatile starting material in isoprenoid synthesis. Both the enantiomers of **59** were prepared by proline-catalyzed asymmetric annelation reaction. The products are unfortunately enantiomerically impure (~70% ee). Although they can be purified by careful fractional recrystallization, this purification is very tedious. Sugai and coworkers devised a simple method for the purification of **59** by kinetic resolution through yeast reduction with *Torulaspora delbrueckii* IFO 10921 (Figure 22.26) [23]. This yeast can reduce only (*S*)-**59** at C-5. Therefore, treatment of (*R*)-**59** of 70% ee with *T. delbrueckii* leaves (*R*)-**59** (97.6% ee) in 82% yield, while (*S*)-**59** of 70% ee gives (4*aS*, 5*S*)-**60** of 94.4% de in 78% yield. Jones chromic acid converts (4*aS*, 5*S*)-**60** to (*S*)-**59**, which can be purified readily to 100% ee by recrystallization.

22.5.2.1 Preparation of (*R*)-**59** [23]

The wet cells of *T. delbrueckii* IFO 10921 (60 g) were suspended in a solution of glucose (20 g), KH_2PO_4 (3.0 g), and K_2HPO_4 (2.0 g) in H_2O (1 L). To this was added (*R*)-**59** of 70% ee (10.0 g, 56.1 mmol), and the mixture was shaken for 6 h at 30°C. The mixture was then filtered through Celite and the filtrate was extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was chromatographed on SiO_2 (450 g, hexane/EtOAc 3:1 to 1:1) to give (*R*)-**59** (8.2 g, 82%) as a solid. This was recrystallized from hexane/EtOAc to give (*R*)-**59** as needles, mp 48.6 to 49.4°C, $[\alpha]_{\text{D}}^{27} = -95.2$ ($c = 1.03$, toluene). Enantiomeric purity determination: HPLC analysis, 99.5% ee.

22.5.2.2 Preparation of (4*aS*, 5*S*)-**60** [23]

The wet cells of *T. delbrueckii* IFO 10921 (60 g) were suspended in a solution of glucose (20 g), KH_2PO_4 (3.0 g), and K_2HPO_4 (2.0 g) in H_2O (1 L). To this was added (*S*)-**59** of 70% ee (10.0 g, 56.1 mmol), and the mixture was shaken for 14 h at 30°C. The mixture was then filtered through Celite and the filtrate was extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was chromatographed on SiO_2 (450 g,

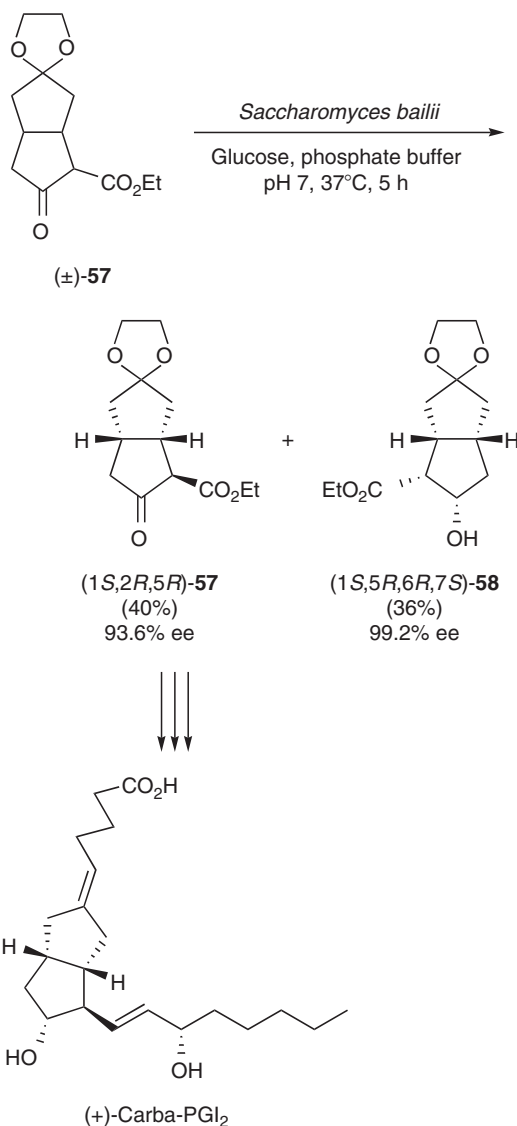


FIGURE 22.25 Preparation of β -keto ester (1*S*,2*R*,5*R*)-**57**.

hexane/EtOAc 3:1 to 1:1) to give (*R*)-**59** (2.2 g, 22%) first, and then (4*aS*,5*S*)-**60** (7.9 g, 78%) as a solid. This was recrystallized from hexane/EtOAc to give needles, mp 44.0 to 46.2°C, $[\alpha]_{\text{D}}^{24} = +191.6$ ($c = 1.05$, benzene). Enantiomeric purity determination: HPLC analysis after oxidation to (*S*)-**59**, >99.9% ee.

22.6 CONCLUSION

We have discussed various biocatalytic processes for preparations of enantiopure building blocks for laboratory organic synthesis. Many new building blocks will be devised in future to facilitate enantioselective synthesis. Three recent reviews are available in this area [43–45].

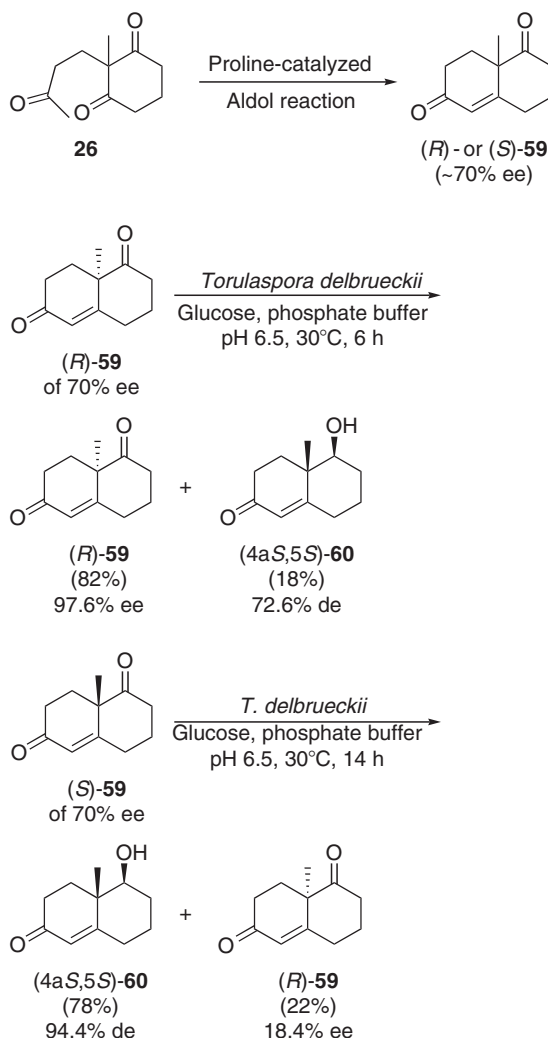


FIGURE 22.26 Purification of Wieland–Miescher ketone **59**.

ACKNOWLEDGMENT

I thank my past and present coworkers whose names appear in References for their experimental efforts and contributions. My thanks are due to Drs. T. Tashiro and K. Fuhshuku for their help in preparing the figures.

REFERENCES

1. Fujita, K. and Mori, K., Synthesis of (2*R*,4*R*)-supellapyrone, the sex pheromone of the brownbanded cockroach, *Supella longipalpa*, and its three stereoisomers, *Eur. J. Org. Chem.*, 493–502, 2001.
2. Nakamura, Y. and Mori, K., Pheromone synthesis, CXCVIII: synthesis of (1*S*,2*S*,6*S*,10*R*)- and (1*S*,2*R*,6*R*,10*R*)-1,2,6,10-tetramethyldodecyl propanoate, the component of the sex pheromone of the pine sawfly, *Microdiprion pallipes*, *Eur. J. Org. Chem.*, 2175–2182, 1999.
3. Chênevert, R. and Desjardins, M., Enzymatic desymmetrization of *meso*-2,6-dimethyl-1,7-heptanediol: enantioselective formal synthesis of the vitamin E side chain and the insect pheromone tribolure, *J. Org. Chem.*, 61, 1219–1222, 1996.

4. Nakamura, Y. and Mori, K., Pheromone synthesis, CCI: synthesis of (3*S*,7*S*)- and (3*S*,7*S*,15*S*)-stereoisomers of 3,7-dimethyl-2-heptacosanone and 3,7,15-trimethyl-2-heptacosanone, the ketones identified from the locust *Schistocerca gregaria*, *Eur. J. Org. Chem.*, 1309–1312, 2000.
5. Nakamura, Y. and Mori, K., New synthesis of the rice moth and stink moth pheromones by employing (2*R*,6*S*)-7-acetoxy-2,6-dimethyl-1-heptanol as a building block, *Biosci. Biotechnol. Biochem.*, 64, 1713–1721, 2000.
6. Seki, M. and Mori, K., Synthesis of a prenylated and immunosuppressive marine galactosphingolipid with cyclopropane-containing alkyl chains: (2*S*,3*R*,11*S*,12*R*,2''*R*,5'''*Z*,11'''*S*,12'''*R*)-plakoside A and its (2*S*,3*R*,11*R*,12*S*,2''*R*,5'''*Z*,11'''*R*,12'''*S*)-isomer, *Eur. J. Org. Chem.*, 3797–3809, 2001.
7. Tashiro, T., Akasaka, K., Ohru, H., Fattorusso, E., and Mori, K., Determination of the absolute configuration at the two cyclopropane moieties of plakoside A, an immunosuppressive marine galactosphingolipid, *Eur. J. Org. Chem.*, 3659–3665, 2002.
8. Grandjean, D., Pale, P., and Chuche, J., Enzymatic hydrolysis of cyclopropanes: total synthesis of optically pure dictyopterene A and C, *Tetrahedron*, 47, 1215–1230, 1991.
9. Brevet, J.-L. and Mori, K., Pheromone synthesis, CXXXIX: enzymatic preparation of (2*S*,3*R*)-4-acetoxy-2,3-epoxybutan-1-ol and its conversion to the epoxy pheromones of the gypsy moth and the ruby tiger moth, *Synthesis*, 1007–1012, 1992.
10. Grandjean, D., Pale, P., and Chuche, J., Synthesis of optically pure *cis* epoxyalcohols via an enzymatic route: an alternative to the Sharpless asymmetric epoxidation, *Tetrahedron Lett.*, 32, 3043–3046, 1991.
11. Mori, K. and Kiyota, H., Pheromone synthesis, CXLII: a new synthesis of (3*S*,4*R*)-8-nonene-3,4-diol, the key intermediate for the synthesis of (+)-*endo*-brevicomin, *Liebigs Ann. Chem.*, 989–992, 1992.
12. Pottie, M., Van der Eycken, J., Vandewalle, M., and Röper, H., The synthesis of optically active derivative of erythritol, *Tetrahedron: Asymmetry*, 2, 329–330, 1991.
13. Mori, K. and Takahashi, Y., Synthetic microbial chemistry, XXIV: synthesis of antibiotic 1233A, an inhibitor of cholesterol biosynthesis, *Liebigs Ann. Chem.*, 1057–1065, 1991.
14. Watanabe, H., Shimizu, H., and Mori, K., Synthesis of compounds with juvenile hormone activity, XXXI: stereocontrolled synthesis of (+)-juvabione from a chiral sulfoxide, *Synthesis*, 1249–1254, 1994.
15. Yanai, M., Sugai, T., and Mori, K., Synthesis of (*S*)-2-hydroxy- β -ionone employing (*S*)-3-hydroxy-2,2-dimethylcyclohexanone as the chiral starting material, *Agric. Biol. Chem.*, 49, 2373–2377, 1985.
16. Mori, K. and Mori, H., Yeast reduction of 2,2-dimethylcyclohexane-1,3-dione: (*S*)-(+)-3-hydroxy-2,2-dimethylcyclohexanone, *Org. Synth. Col. Vol.*, 8, 312–315, 1993.
17. Watanabe, H. and Mori, K., Triterpenoid total synthesis, Part 2: synthesis of glycinoeclepin A, a potent hatching stimulus for the soybean cyst nematode, *J. Chem. Soc. Perkin Trans. 1*, 2919–2934, 1991.
18. Mori, K. and Mori, H., Synthesis of both the enantiomers of juvenile hormone III, *Tetrahedron*, 43, 4097–4106, 1987.
19. Mori, K. and Nagano, E., Preparative bioorganic chemistry, Part 10: asymmetric reduction of bicyclo[2.2.2]octane-2,6-diones with baker's yeast, *Biocatalysis*, 3, 25–36, 1990.
20. Mori, K. and Matsushima, Y., Synthesis of mono- and sesquiterpenoids, XXII: synthesis of (+)-pinthunamide, a sesquiterpene metabolite of a fungi, *Ampulliferina* sp., *Synthesis*, 406–410, 1993.
21. Mori, K. and Fujiwhara, M., Synthesis of enantiomerically pure (10*R*,11*S*)-(+)-juvenile hormones I and II, *Tetrahedron*, 44, 343–354, 1988.
22. Nagano, E. and Mori, K., Synthesis of (+)-juvabione, a compound with juvenile hormone activity, *Biosci. Biotechnol. Biochem.*, 56, 1589–1591, 1992.
23. Fuhshuku, K., Funai, N., Akeboshi, T., Ohta, H., Hosomi, H., Ohba, S., and Sugai, T., Access to Wieland–Miescher ketone in an enantiomerically pure form by a kinetic resolution with yeast-mediated reaction, *J. Org. Chem.*, 65, 129–135, 2000.
24. Fuhshuku, K., Tomita, M., and Sugai, T., Enantiomerically pure octahydronaphthalenone and octahydroindenone: elaboration of the substrate overcame the specificity of yeast-mediated reduction, *Adv. Synth. Catal.*, 345, 766–774, 2003.
25. Nishimura, Y. and Mori, K., Pheromone synthesis, 188: a new synthesis of (–)-frontalin, the bark beetle pheromone, *Eur. J. Org. Chem.*, 233–236, 1998.

26. Kitahara, T. and Mori, K., Preparation of chiral cyclohexanol derivative with high optical purity by yeast reduction, *Tetrahedron Lett.*, 26, 451–452, 1985.
27. Kitahara, T., Kurata, H., and Mori, K., Efficient synthesis of the natural enantiomer of sporogen-AO 1 (13-desoxyphomenone), a sporogenic sesquiterpene from *Aspergillus oryzae*, *Tetrahedron*, 44, 4339–4349, 1988.
28. Muto, S. and Mori, K., Synthesis of all four stereoisomers of leucomalure, components of the female sex pheromone of the satin moth, *Leucoma salicis*, *Eur. J. Org. Chem.*, 1300–1307, 2003.
29. Tashiro, T. and Mori, K., Enzyme-assisted synthesis of (*S*)-1,2-dihydroxy-3,7-dimethyl-6-octen-2-one, the male-produced aggregation pheromone of the Colorado potato beetle, and its (*R*)-enantiomer, *Tetrahedron: Asymmetry*, 16, 1801–1806, 2005.
30. Horiuchi, S., Takikawa, H., and Mori, K., Enzyme resolution of (\pm)- γ -cyclohomogeneranol and conversion of its (*S*)-isomer to (*S*)- γ -coronal, the ambergris odorant, *Bioorg. Med. Chem.*, 7, 723–726, 1999.
31. Horiuchi, S., Takikawa, H., and Mori, K., Synthesis of (6*S*,7*S*)-7-hydroxy-6,11-cyclofarnes-3(15)-en-2-one, the opposite enantiomer of the antibacterial sesquiterpene from *Premna oligotricha*, and the (*R*)-enantiomer of ancistrodial, the defensive sesquiterpene from *Ancistrotermes cavithorax*, *Eur. J. Org. Chem.*, 2851–2854, 1998.
32. Mori, K. and Puapoomchareon, P., Preparative bioorganic chemistry, XV: preparation of optically pure 2,4,4-trimethyl-2-cyclohexen-1-ol, a new and versatile chiral building block in terpene synthesis, *Liebigs Ann. Chem.*, 1053–1056, 1991.
33. Mori, K., Amaike, M., and Itou, M., Synthesis of (*S*)- α -damascone, *Tetrahedron*, 49, 1053–1056, 1993.
34. Mori, K. and Khlebnikov, V., Carotenoids and degraded carotenoids, VIII: synthesis of (+)-dihydroactinidiolide, (+)- and (–)-actinidiolide, (+)- and (–)-loliolide as well as (+)- and (–)-epiloliolide, *Liebigs Ann. Chem.*, 77–82, 1993.
35. Hirayama, K. and Mori, K., Plant bioregulators, 5: synthesis of (+)-strigol and (+)-orobanchol, the germination stimulants, and their stereoisomers by employing lipase-catalyzed asymmetric acetylation as the key step, *Eur. J. Org. Chem.*, 2211–2217, 1999.
36. Takikawa, H., Nozawa, D., Kayo, A., Muto, S., and Mori, K., Synthesis of sphingosine relatives, Part 22: synthesis of sulfobacin A,B and flavocristamide A, new sulfolipids isolated from *Chryseobacterium* sp., *J. Chem. Soc. Perkin Trans. 1*, 2467–2477, 1999.
37. Masuda, Y., Yoshida, M., and Mori, K., Synthesis of (2*S*,2'*R*,3*S*,4*R*)-2-(2'-hydroxy-21'-methyldocosanoylamino)-1,3,4-pentadecanetriol, the ceramide sex pheromone of the female hair crab, *Erimacrus isenbeckii*, *Biosci. Biotechnol. Biochem.*, 66, 1531–1537, 2002.
38. Bakke, M., Takizawa, M., Sugai, T., and Ohta, H., Lipase-catalyzed enantiomer resolution of ceramides, *J. Org. Chem.*, 63, 6929–6938, 1998.
39. Yokoyama, Y., Takikawa, H., and Mori, K., Enzymatic preparation of (4*R*,6*R*)-4-hydroxy-1,7-dioxaspiro[5.5]undecane and its antipode, the minor component of the olive fruit fly pheromone, *Bioorg. Med. Chem.*, 4, 409–412, 1996.
40. Ichihashi, M., Takikawa, H., and Mori, K., Synthesis of (*R*)-(+)-hippospongic acid A, a triterpene isolated from the marine sponge, *Hippospongia*, sp., *Biosci. Biotechnol. Biochem.*, 65, 2569–2572, 2001.
41. Mori, K. and Funaki, Y., Synthesis of (4*E*,8*E*,2*S*,3*R*,2'*R*)-*N*-2'-hydroxyhexadecanoyl-9-methyl-4,8-sphingadienine, the ceramide portion of the fruiting-inducing cerebroside in a basidiomycete *Schizophyllum commune*, *Tetrahedron*, 41, 2369–2377, 1985.
42. Mori, K. and Tsuji, M., A new synthesis of (+)-6 α -carbaprostaglandin I₂ employing yeast reduction of a β -keto ester derived from *cis*-bicyclo[3.3.0]octane-3,7-dione as the key step, *Tetrahedron*, 42, 435–444, 1986.
43. Fuhshuku, K., Oda, S., and Sugai, T., Enzyme reactions as the key steps in the synthesis of terpenoids, degraded carotenoids, steroids, and related substances, *Recent Res. Devel. Org. Chem.*, 6, 57–75, 2002.
44. Brenna, E., Enzyme-mediated synthesis of chiral communication substances: fragrances for perfumery applications, *Curr. Org. Chem.*, 7, 1347–1367, 2003.
45. Ghanem, A. and Aboul-Enen, H.Y., Lipase-mediated chiral resolution of racemates in organic solvents, *Tetrahedron: Asymmetry*, 15, 3331–3351, 2004.

23 Stereoselective Modifications of Polyhydroxylated Steroids

Elena Fossati and Sergio Riva

CONTENTS

23.1	Introduction	591
23.2	Regio- and Stereoselective Oxidoreductions Catalyzed by HSDH	592
23.3	Regioselective Modification of Polyhydroxylated Steroids Catalyzed by Lipases and Proteases	595
23.3.1	Regioselective Esterification of Hydroxyl Groups Located on the “A” Ring	597
23.3.2	Regioselective Esterification of Hydroxyl Groups Located on the “D” Ring or on the Side Chain	598
23.3.3	Regioselective Enzymatic Alcoholysis of Polyacylated Hydroxysteroids	599
23.4	Conclusion	601
	References	602

23.1 INTRODUCTION

Nature is a tireless architect of complex chemical structures that can quite often be exploited to improve human health and well-being. Among them, steroids are probably the most widely investigated family of bioactive compounds. Specifically, their typical tetracyclic rigid structure decorated with a bunch of sensitive functional groups has attracted the synthetic interest of organic chemists, who have always regarded these molecules as ideal targets to develop or to apply new selective reactions [1–4]. Similarly, biocatalyzed transformations of steroids—by whole-cell processes [5–6] or by isolated enzyme-catalyzed reactions [7]—have been widely investigated, making available an enormous number of scientific papers, published in both chemical and biological journals.

Among these data, two kinds of stereoselective transformations of polyhydroxylated steroids will be discussed:

- (1) The regio- and stereoselective oxidoreductions catalyzed by hydroxysteroid dehydrogenases (HSDH)
- (2) The regioselective acylation catalyzed by lipases and proteases

An important parameter to be considered for these efficient enzymatic transformations is the reaction solvent. On this respect, different approaches, such as water, biphasic systems, and organic solvents, will be discussed.

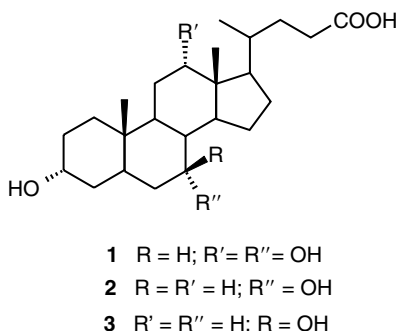


FIGURE 23.1 Compounds 1–3

23.2 REGIO- AND STEREOSELECTIVE OXIDOREDUCTIONS CATALYZED BY HSDH

In nature, the regio- and stereoselective interconversions of secondary alcohols to ketones on the steroid nucleus and side chain are catalyzed by HSDH, a group of NAD(P)-dependent oxidoreductases [8].

The synthetic exploitation of these enzymes was initially described with bile acids [9]. These steroids are derivatives of 5 β -cholan-24-oic acid and represent the major quantitative pathway by which cholesterol is metabolized in the human body. Cholic acid (**1**, Figure 23.1) and chenodeoxycholic acid (**2**) are the primary bile acids in human bile; the latter compound and its 7 β -hydroxy epimer, ursodeoxycholic acid (**3**), have important pharmaceutical applications related to their ability to solubilize cholesterol gallstones. Thanks to their carboxylic group, bile acids are water-soluble at mild alkaline pH and therefore enzymatic oxidoreductions on these compounds are mainly performed in aqueous solutions. In these examples, the *in situ* regeneration of the nicotinamide cofactors was obtained by coupled enzymatic reactions that allowed the use of catalytic amounts of NAD(P)(H) [10].

As a first example, Figure 23.2 shows the regio- and stereospecific reductions of dehydrocholic acid (**4**) with different HSDH [11,12]. The reduced products were obtained quantitatively and, by using a suitable deuterated substrate donor for cofactor regeneration (i.e., 1-D-glucose and glucose dehydrogenase), it was also possible to synthesize selectively deuterated steroids [13]. Similarly, quantitative regioselective oxidation of cholic acid (**1**) was also described [11,14], and, in a recent example, 12 α -HSDH was used for the preparative-scale oxidation of Gd(III) complexes of ligands containing derivatives of 7,12-dihydroxy or 12-hydroxy cholanoic acids [15].

In a more general approach, the hydroxyl α/β inversion at position 3 and 7 of the steroid skeleton was obtained in a two-step enzymatic process (Figure 23.3) [16–18].

HSDHs are also active on neutral steroids. To overcome the very low water solubility of these compounds, new and innovative (by those times) two-phase systems [19] were described in the late 1970s, the reaction conditions being optimized with a series of androstane and pregnane derivatives [20–23].

Figure 23.4 summarizes the industrial chemical route for the transformation of cholic acid (**1**) into the bioactive derivative ursodeoxycholic acid (**3**). Compound **5**, 12-ketochenodeoxycholic acid, is a key intermediate and can be prepared enzymatically from **1** in just one-step by using 12 α -HSDH [11,24,25]. Following optimization of the reaction conditions, compound **5**

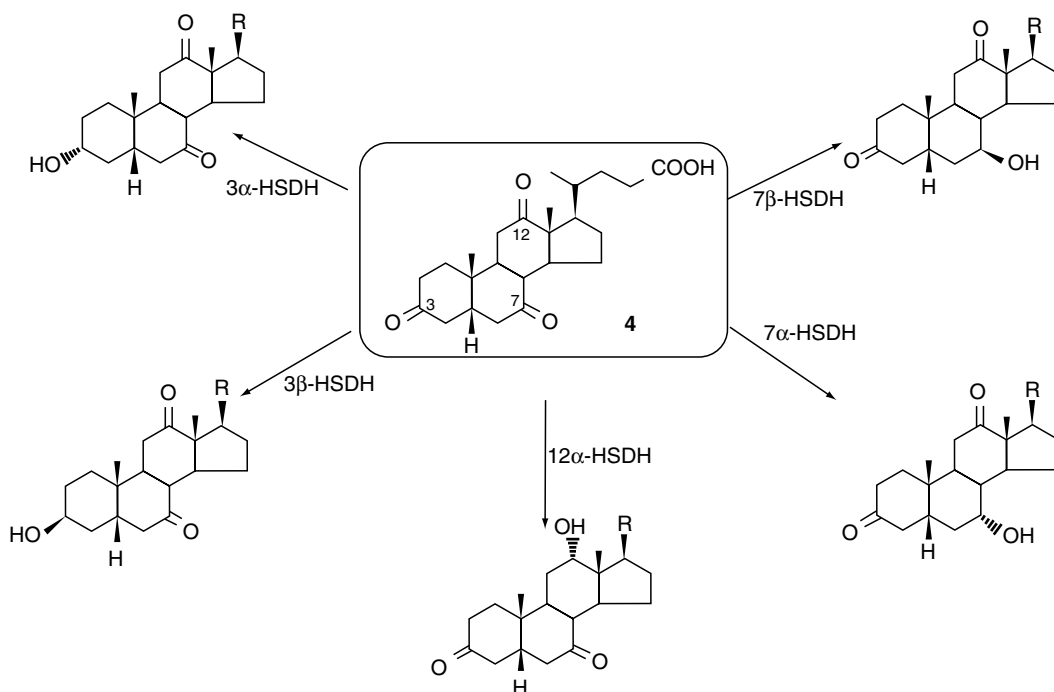


FIGURE 23.2 Regio- and stereospecific reduction of dehydrocholic acid (**4**) catalyzed by different hydroxysteroid dehydrogenases.

was obtained on a multikilogram scale in a membrane reactor containing an initial 4% w/v solution of cholic acid (**1**). Free 12 α -HSDH and glutamate dehydrogenase, the enzyme used to regenerate NADP by reduction of α -ketoglutarate to glutamate, were retained inside the reactor and were reused for several cycles (up to 50) with satisfactory retention of their catalytic activity.

Recently, a different NADP-regeneration system, based on the use of alcohol dehydrogenases together with a large excess of acetone, has been discussed [26].

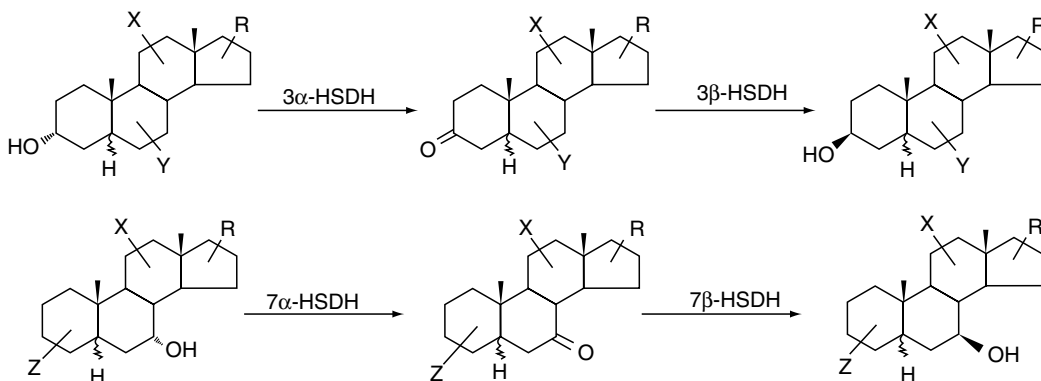


FIGURE 23.3 3 α /3 β -OH and 7 α /7 β -OH inversions catalyzed by hydroxysteroid dehydrogenases.

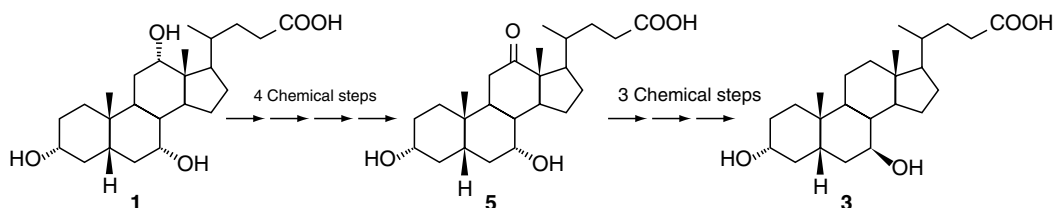


FIGURE 23.4 Chemical synthesis of ursodeoxycholic acid (3) from cholic acid (1).

Alternative chemoenzymatic routes to ursodeoxycholic acid were also investigated. Figure 23.5 shows a three-step sequence that involves two chemical and one enzymatic transformation. Preparation of 12-chetoursodeoxycholic acid (6) from dehydrocholic acid (4) was obtained in one step by using in the same reactors 3 α -HSDH (NAD dependent), 7 β -HSDH (NADP dependent), and glucose dehydrogenase (to regenerate both NADH and NADPH) [27].

In an alternative three-step sequence, compound 3 was obtained by a chemical reduction of 6, (Figure 23.6) prepared from cholic acid through two consecutive enzymatic steps [28].

The scale-up of dehydrogenase-catalyzed reactions can theoretically be performed in different reactors, using free or immobilized enzymes, neutral or charged membranes, and native or modified nicotinamide cofactors. The general goal is obviously to recycle the enzymes—that are retained inside the reactor—and, if possible, the expensive coenzyme. In this respect, an interesting process was developed by the immobilization of native dehydrogenases

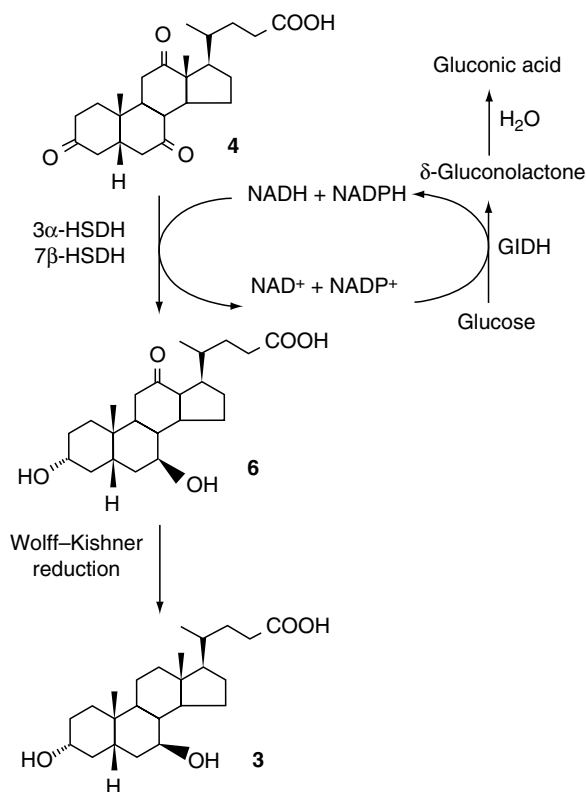


FIGURE 23.5 Proposed three-step chemoenzymatic route to ursodeoxycholic acid (3).

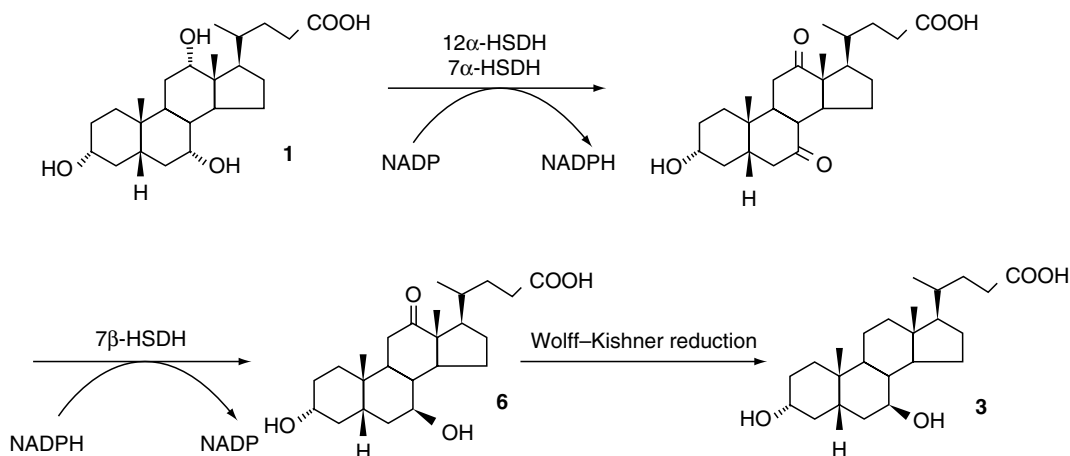


FIGURE 23.6 Alternative three-step chemoenzymatic route to ursodeoxycholic acid (3).

into isoelectric traps, formed by pairs of isoelectric membranes encompassing their pI values. The enzyme (3β-HSDH) was forced to perform its catalytic activity in an electric field coupled orthogonally to a hydraulic flow, which allowed the continuous transportation and harvesting of the charged bile acid product, by voltage gradient, into neighboring chambers [29].

The analytical exploitation of HSDHs for the quantitative enzymatic determination of neutral steroids and bile acid derivatives in serum or other physiological fluids has been reported [30–33]. Evaluation of steroid concentration was performed by spectrophotometric, fluorometric, and bioluminescence assays. The high sensitivity of the latter method allowed the detection of as little as picomoles of steroids using enzymes coimmobilized on nylon tubes [33].

23.3 REGIOSELECTIVE MODIFICATION OF POLYHYDROXYLATED STEROIDS CATALYZED BY LIPASES AND PROTEASES

Enzyme catalysis in organic solvents is a well-assessed methodology [34,35]. Specifically, when suspended in organic solvents hydrolytic enzymes, i.e., lipases and proteases, can efficiently catalyze stereoselective transesterification, esterification, aminolysis, acyl exchange, and thio-transesterification reactions [36], transformations that in water or biphasic systems are suppressed by the “natural” hydrolytic activity and therefore, do not occur to appreciable extent.

Lipase- and protease-catalyzed regioselective esterifications of sugars were initially achieved [37,38], but the methodology was soon extended to other polyhydroxylated natural compounds, including hydroxysteroids.

In a first report [39], two hydrolases—the lipase from *Chromobacterium viscosum* (*Chv*) and the protease subtilisin—were found to acylate the model dihydroxysteroid 5α-androstane-3β,17β-diol (7) in acetone with opposite regioselectivity; while *Chv* lipase reacted exclusively with the C-3 OH, subtilisin displayed a preference for the C-17 OH (Figure 23.7, the chemical reactivities of C-3 OH and C-17 OH were comparable). The selectivity of these two enzymes was examined with other hydroxysteroids and the results are reported in Table 23.1. Only C-3 OHs in the equatorial (β) position of steroids having a *trans* A/B ring fusion (or a C-5 to C-6 double bond) were acylated by *Chv* lipase, while changes in the A or B rings did not dramatically affect the reactivity of the steroid with subtilisin.

Later, two other lipases were found to be able to acylate hydroxysteroids, the lipase from *Candida cylindracea* (*Cac*, subsequently identified as *C. rugosa* lipase, *Car*, Figure 23.7) [40]

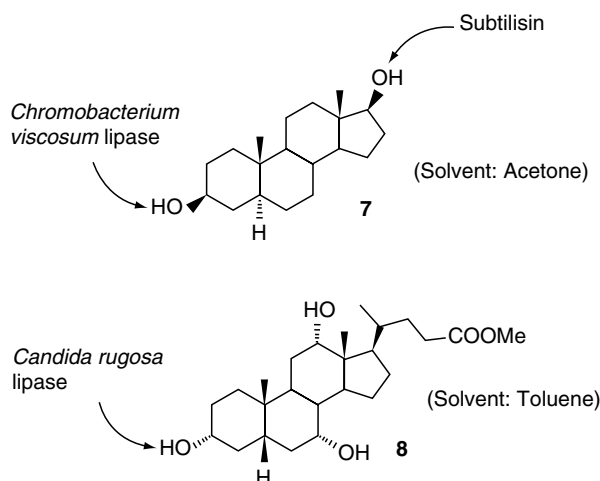
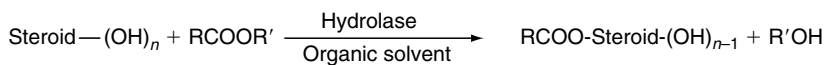


FIGURE 23.7 Regioselective lipase-catalyzed acylation of polyhydroxylated steroids.

and the lipase B from *C. antarctica* (*CaaB*, also known as Novozym 435) [41], and their selectivities were evaluated. The former enzyme was effective in the esterification of C-3 α OHs of bile acid methyl esters (*cis* A/B ring fusion, i.e., **8**), while the latter one was active both on C-3 OHs and on side chain OHs. These reports, as well as further data published recently, indicate that no enzymes are able to acylate hydroxyl groups located on the inner B and C rings, while esterification of OHs on the A and D rings, as well as on the side chain, can be obtained by a proper choice of the right hydrolase.

TABLE 23.1

Reactivities of Various Hydroxysteroids in the Acylation Catalyzed by *Chromobacterium viscosum* Subtilisin and Lipase Suspended in Acetone

Steroid	Initial Rate ($\mu\text{mol/h}$)	
	Subtilisin	Lipase
3 β ,17 β -Dihydroxy-5 α -androstane	0.63	3.30
3 α ,17 β -Dihydroxy-5 α -androstane	0.53	0
3 β ,17 β -Dihydroxy-5 β -androstane	0.41	0
3 α ,17 β -Dihydroxy-5 β -androstane	0.63	0
3 α ,17 β -Dihydroxy-5 α -androstene	0.67	1.72
3 α ,17 β -Dihydroxy-4-androstene	0.32	0
17 β -Estradiol	0.63	0
3 β -Hydroxy-5 α -pregnane	0.06	6.26
3 β -Hydroxy-5 α -cholanolic acid methyl ester	0	8.32
3 β -Hydroxy-5 α -cholenic acid methyl ester	0	2.75
3 β -Hydroxy-5 α -cholestene (cholesterol)	0	3.00
3 β ,20 β -Dihydroxy-5 α -pregnane	0.10	4.52
3 β ,20 α -Dihydroxy-5 α -pregnane	0.55	4.34

23.3.1 REGIOSELECTIVE ESTERIFICATION OF HYDROXYL GROUPS LOCATED ON THE "A" RING

Monoacylated derivatives of a complete set of 2,3- and 3,4-vicinal diols of steroids were recently prepared by regioselective lipase-catalyzed transesterifications [42]. As expected, different lipases (*CaaB*, *Car*, *Chy*, along with the lipase PS from a *Pseudomonas* strain) displayed different selectivities toward the vicinal diols depending on the OHs configuration. The results are shown in Figure 23.8, which shows that the acylation mainly took place at the C-3 position (as expected), except by lipase PS (esterification of C-4 α OH in **14** and of C-2 β OH in **15**).

In another paper, the same authors obtained stereoisomerically pure 3 β -hydroxy-5,6-epoxysteroids by combining selective chemical methods for α - and β -epoxidation of Δ^5 -unsaturated steroids with enzymatic stereoselective esterification of their 3 β -OHs. Specifically, 3 β -OH-5 β ,6 β -epoxysteroids were acylated by *CaaB* and by the lipase AK (from a *Pseudomonas* strain), while 3 β -OH-5 α ,6 α -epoxysteroids were good substrates for *Car* (Figure 23.9) [43].

A similar approach was used by Santaniello and coworkers for the chemoenzymatic synthesis of the 3-hydroxy metabolites of the estrogen tibolone (**22**, Figure 23.10): *CaaB* catalyzed the selective acetylation of the 3 β -OH epimer of **22** [44]. The peculiar regioselectivity of this lipase was also exploited by the same authors in an elegant preparation of oxandrolone (**23**), an anabolic synthetic hormone that was capable of improving the quality of life for patients with HIV-infections [45].

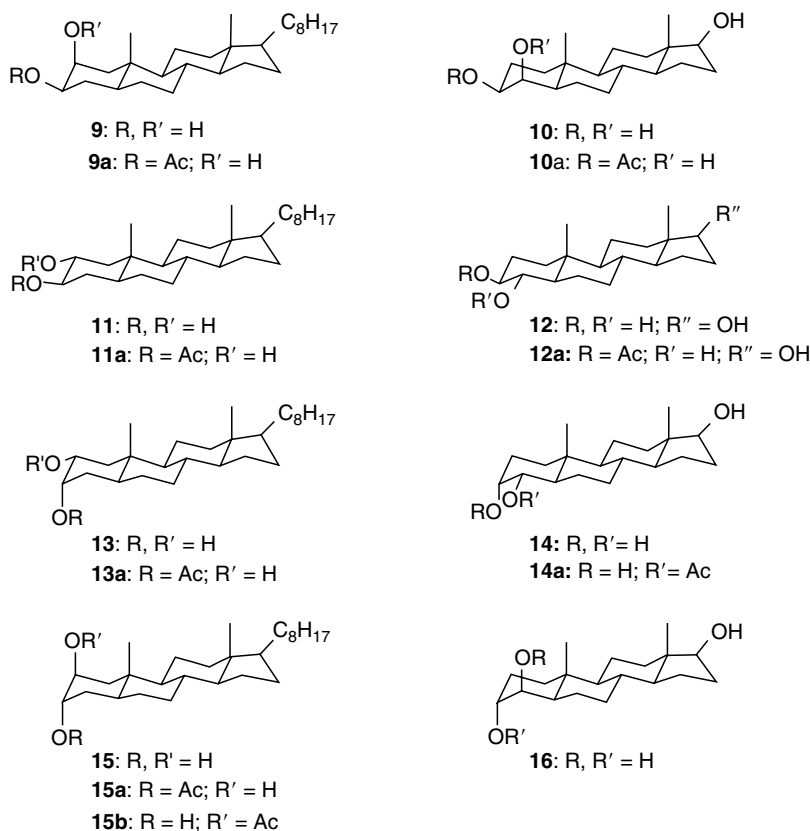


FIGURE 23.8 Regioselective lipase-catalyzed acylation of steroid diols.

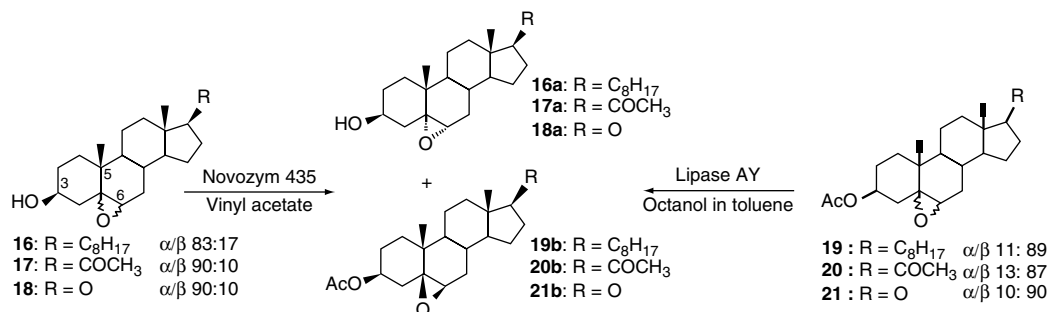


FIGURE 23.9 *CaaB* lipase-catalyzed stereoselective esterification of 3β-acetoxy-5,6-epoxysteroids and *Car* lipase-catalyzed stereoselective alcoholysis of 3β-acetoxy-5,6-epoxysteroids.

Regioselective esterification of a series of ecdysteroids (like 20*R*-hydroxyecdisonone, **24**) was reported in the late 1990s [46]; despite the numerous OH groups present on these molecules, *CaaB* directed its action exclusively on the C-2 OH.

The lipase-catalyzed regioselective preparation of 3-hemisuccinates of polyhydroxylated steroids [47] and of biologically active esters of dehydroepiandrosterone deserve also to be reported [48].

23.3.2 REGIOSELECTIVE ESTERIFICATION OF HYDROXYL GROUPS LOCATED ON THE “D” RING OR ON THE SIDE CHAIN

As discussed previously, the most interesting results reported in the first paper on the enzymatic acylation of steroids [39] was the complementary regioselectivity of the used hydrolases, with a clear indication of the preference of the protease subtilisin for the OHs located on the D ring or on the side chain. Additionally, the data reported in the last two lines of Table 23.1 show that this protease was also sensitive to the stereochemistry of the nucleophile, such as in cases 20-OH epimers belonging to the pregnane family (the 20α-epimer being the preferred one).

Lipases do not acylate hydroxyl moieties on the D ring, but can act on steroids side chain. For instance, a primary C-21 OH of a pregnene derivative was an excellent substrate for *CaaB* [41], and the same enzyme also showed a stereopreference for C-20 OHs: the 20β-epimer was acylated while the 20α-OH was not (a result complementary to the one obtained with subtilisin). It is known that lipases and proteases usually work with opposite enantioselectivity).

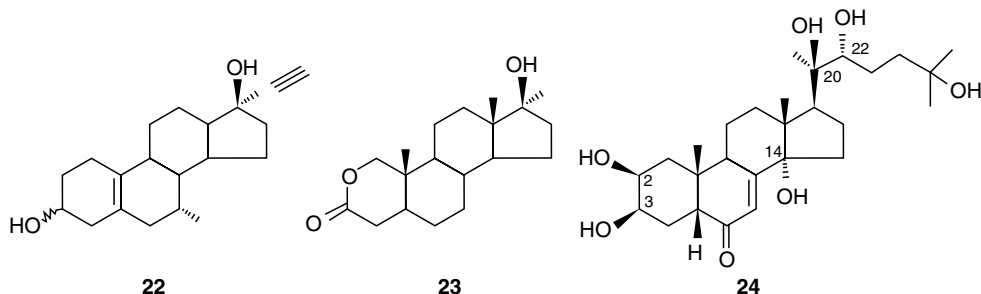


FIGURE 23.10 Compounds 22–24.

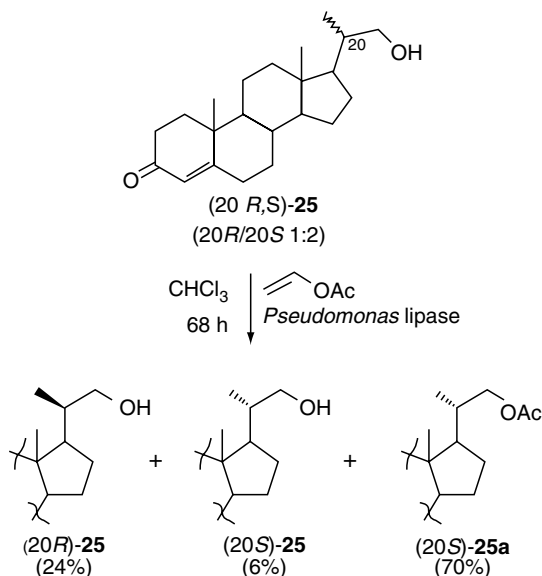


FIGURE 23.11 Stereoselective acylation of **22**.

In addition to the previously reported scant data, acylation of hydroxyl groups in steroid side chains was investigated in detail by Santaniello and coworkers and their results are reported in a series of papers [49–53]. For instance, Figure 23.11 shows the stereoselective acylation of the (20*S*)-isomer of the C-22 OH in compound **25** by action of a lipase from *Pseudomonas* [49].

Recently, the steroidal cyanohydrin derivative **26** could be isolated with a 89% d.e. through the subtilisin-catalyzed acylation of an epimeric mixture of the parent cyanohydrin [54].

23.3.3 REGIOSELECTIVE ENZYMIC ALCOHOLYSIS OF POLYACYLATED HYDROXYSTEROIDS

Hydrolases regioselectivity can also be exploited to get the mild removal of a protecting ester moiety, thus avoiding the negative drawbacks of classical chemical saponifications: lack of selectivity and promotion of undesirable side reactions. The application of this methodology to steroids was pioneered by Njar and Caspi in the late 1980s [55]. They showed that *Car* lipase suspended in isopropyl ether or in acetonitrile in the presence of *n*-octanol catalyzed the alcoholysis of different diacetylated steroids to give octyl acetate and monoacetylated steroids.

These transformations were later investigated in more detail by Baldessari and coworkers [56–58]. In a first report, they screened several lipases and proteases for the mild alcoholysis of the 3 β -acetoxy derivatives **27** (a model compound) and **28**, evaluating the influence of the nature of the organic solvent and of the alcohol on the efficiency of the reaction. *Car* and *CaaB* lipases were the only active catalysts, toluene and acetonitrile were the best solvents, respectively, and octanol was the best nucleophile with both enzymes [56]. Under the best conditions the diol **28a** was isolated in 68% yields, without altering the highly sensitive 20 β -ketol system that might easily undergo cleavage to give the 20-ketopregnene derivative (Figure 23.12).

The performances of the same enzymes were evaluated with the di- and triacetylated derivatives **29–34**, and the results were quite surprising [57]. While *Car* lipase gave the expected 3 β -OH derivatives **28a–34a**, the lipase from *C. antarctica* directed its action to the

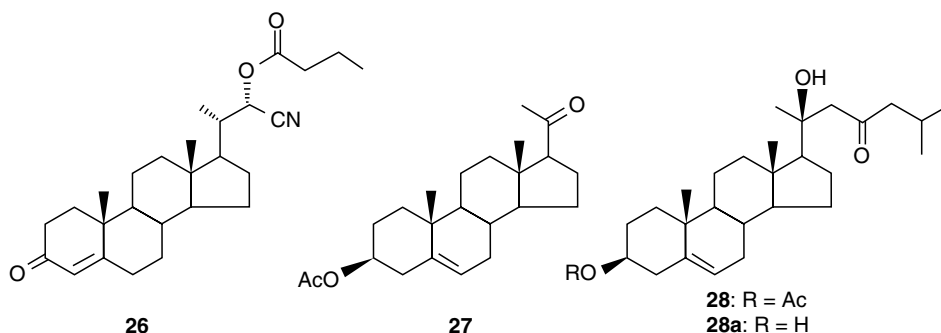


FIGURE 23.12 Compounds **26–28**.

16 β -OAc of compounds **28–30** and to the 17-OAc of **31**, to give the corresponding monoacetates **29b–31b**. Compounds **32–34** were poor substrates for the same enzyme, and with these compounds its action was directed toward the 3 β -OAc. A similar behavior of these two enzymes was observed with another series of androstane and pregnane polyacetylated derivatives (i.e., **35**), and in the publication, additional information on the regioselectivity of the lipase from *Pseudomonas* on these new substrates was reported (Figure 23.13) [58].

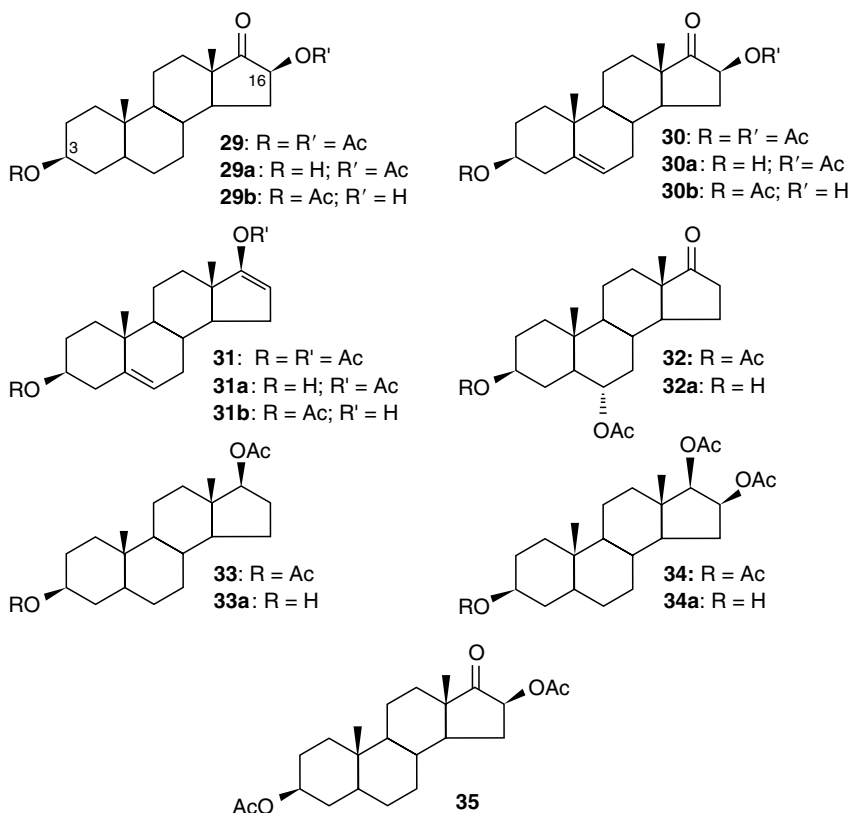


FIGURE 23.13 Compounds **29–35**.

Synthetic exploitation of enzymatic alcoholysis reactions was also described in two of the previously cited reports, for the preparation of oxandrolone [45] and diastereomeric 3 β -5,6-epoxysteroids [43].

23.4 CONCLUSION

It has been shown that oxidoreductases and hydrolases are efficient catalysts for the selective modification of polyfunctionalized steroids. Needless to say, their action can be combined to increase the number of available derivatives. This point has been well-exemplified by Secundo and coworkers, who obtained a library of cholic acid methyl ester derivatives by alternating a stereo- and regioselective oxidative step, catalyzed by HSDH, and an acylation step with a series of different acyl donors (Figure 23.14), catalyzed by *C. antarctica* lipase B [59]. In the cited example, only about one third of the enzymatic reactions tested were productive, nevertheless a 39-member collection of bile acid derivatives (some of which are reported in Figure 23.15) was obtained in high purity and in good yields.

In conclusion, even when focusing on a small group of natural compounds (in this specific case polyhydroxylated steroids), it is possible to highlight the enormous opportunities that biocatalysis offers to organic chemists. Specifically, it has been shown that the highly

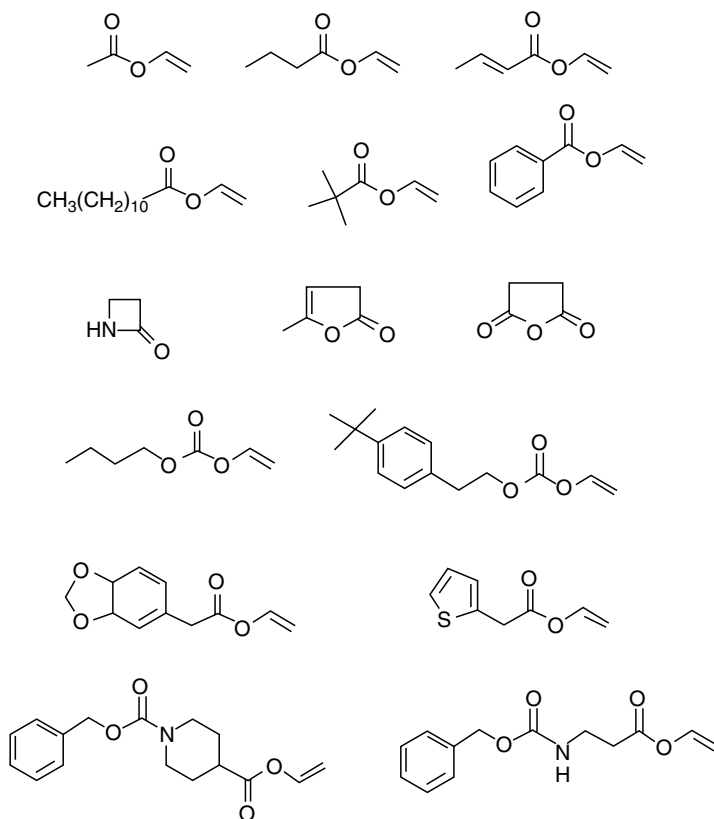


FIGURE 23.14 Examples of activated esters employed in the acylation step.

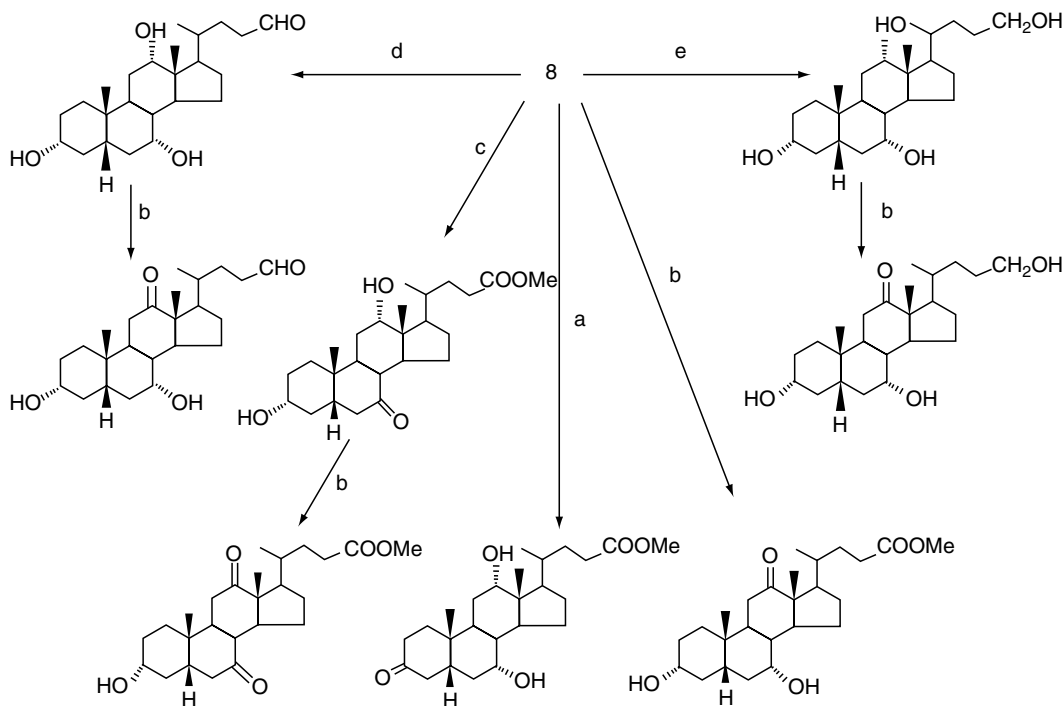


FIGURE 23.15 Example of combinatorial biocatalysis on polyhydroxylated steroids. (a–c) HSDHs-catalyzed oxidations; (d–e) chemical reductions.

substrate-specific enzymes HSDH are very close to industrial-scale exploitation and it pointed out the surprisingly wide, apparently never-ending, synthetic solutions that lipases and proteases in organic solvents might offer to scientific investigators.

REFERENCES

1. Fieser, L.F. and Fieser, M., Eds., *Steroids*, Reinhold, New York, 1959.
2. Djerassi, C., Ed., *Steroid Reactions, An Outline for Organic Chemists*, Holden-Day, San Francisco, CA, 1963.
3. Fried, J. and Edwards, J.A., Eds., *Organic Reactions in Steroid Chemistry*, Reinhold, New York, 1972.
4. Blickenstaff, R.T., Ghosh, A.C., and Wolf, G.C., Eds., *Total Synthesis of Steroids*, Academic Press, New York, 1974.
5. Charney, W. and Herzog, H.L., *Microbial Transformation of Steroids*, Charney, W. and Herzog, H.L., Eds., Academic Press, New York, 1967.
6. Iisuka, K. and Naito, A., Eds., *Microbial Conversion of Steroids and Alkaloids*, Springer, New York, 1981.
7. Riva, S., Enzymatic modification of steroids, in *Applied Biocatalysis*, vol. 1, Blanch, H.W. and Clark, D.S., Eds., Marcel Dekker, New York, pp. 179–220, 1991.
8. Maser, E., Xenobiotic carbonyl reduction and physiological steroid oxidoreduction: the pluri-potency of several hydroxysteroid dehydrogenases, *Biochem. Pharmacol.*, 49(4), 421–440, 1995.
9. Bortolini, A., Medici, A., and Poli, S., Biotransformations on steroid nucleus of bile acids, *Steroids*, 62, 564–577, 1997.
10. Chenault, H.K. and Whitesides, G.M., Regeneration of nicotinamide cofactors for use in organic synthesis, *Appl. Biochem. Biotechnol.*, 14, 147–197, 1987.

11. Riva, S., Bovara, R., Pasta, P., et al., Preparative-scale regio- and stereospecific oxidoreduction of cholic acid and dehydrocholic acid catalyzed by hydroxysteroid dehydrogenase, *J. Org. Chem.*, 51, 2902–2906, 1986.
12. Carrea, G., Bovara, R., Longhi, R., et al., Enzymatic reduction of dehydrocholic acid to 12-ketochenodeoxycholic acid with NADH regeneration, *Enzyme Microb. Technol.*, 6, 307–311, 1984.
13. Riva, S., Ottolina, G., Carrea, G., et al., Efficient preparative-scale enzymatic synthesis of specifically deuterated bile acids, *J. Chem. Soc. Perkin Trans. I*, 2073–2074, 1989.
14. Bianchini, E., Chinaglia, N., Dean, M., et al., Regiospecific oxidoreductions catalyzed by a new *Pseudomonas paucimobilis* hydroxysteroid dehydrogenase, *Tetrahedron*, 55, 1391–1398, 1999.
15. Anelli, P.L., Brocchetta, M., Morosini, P., et al., Conjugates of Gd(III) complexes to di- and tri-hydroxy substituted cholanoic acids: regioselective oxidation with hydroxysteroid dehydrogenases, *Biocatalysis Biotrans.*, 20, 29–34, 2002.
16. Riva, S., Bovara, R., Zetta, L., et al., Enzymatic α/β inversion of C-3 OH of bile acids and study of the effects of organic solvents on reaction rates, *J. Org. Chem.*, 53, 88–92, 1988.
17. Bovara, R., Canzi, E., Carrea, G., et al., Enzymatic α/β inversion of the C-7-hydroxyl of steroids, *J. Org. Chem.*, 58, 499–501, 1993.
18. Medici, A., Pedrini, P., Bianchini, E., et al., 7α -Epimerization of bile acids via oxido-reduction with *Xanthomonas maltophilia*, *Steroids*, 67, 51–56, 2002.
19. Carrea, G., Biocatalysis in water–organic solvent two-phase systems, *Trends Biotech.*, 2(4), 102–106, 1984.
20. Carrea, G. and Cremonesi, P., Enzyme catalyzed transformations in water–organic solvent two-phase systems, in *Methods in Enzymology*, vol. 136, Mosbach, K., Ed., Academic Press, New York, pp. 150–157, 1987.
21. Cremonesi, P., Carrea, G., Sportoletti, G., et al., Enzymatic dehydrogenation of steroids by β -hydroxysteroid dehydrogenase in a two-phase system, *Arch. Biochem. Biophys.*, 159, 7–10, 1973.
22. Cremonesi, P., Carrea, G., Ferrara, L., et al., Enzymatic preparation of 20β -hydroxysteroids in a two-phase system, *Biotechnol. Bioeng.*, 17, 1101–1108, 1975.
23. Carrea, G., Riva, S., Bovara, R., et al., Enzymatic oxidoreduction of steroids in two-phase systems: effects of organic solvents on enzyme kinetics and evaluation of the performance of different reactors, *Enzyme Microb. Technol.*, 11, 333–340, 1988.
24. Carrea, G., Bovara, R., Cremonesi, P., et al., Enzymatic preparation of 12-ketochenodeoxycholic acid with NADP regeneration, *Biotechnol. Bioeng.*, 26, 560–563, 1984.
25. Carrea, G., Bovara, R., Longhi, R., et al., Preparation of 12-ketochenodeoxycholic acid using coimmobilized 12α -hydroxysteroid dehydrogenase and glutamate dehydrogenase with NADP cycling at high efficiency, *Enzyme Microb. Technol.*, 7, 597–600, 1985.
26. Fossati, E., Polentini, F., Carrea, G., et al., Exploitation of the alcohol dehydrogenase-acetone NADP regeneration system for the enzymatic preparative-scale production of 12-ketochenodeoxycholic acid, *Biotechnol. Bioeng.*, 93, 1216–1220, 2006.
27. Carrea, G., Pilotti, A., Riva, S., et al., Enzymatic synthesis of 12-ketoursodeoxycholic acid from dehydrocholic acid in a membrane reactor, *Biotechnol. Lett.*, 14, 1131–1135, 1992.
28. Bovara, R., Carrea, G., Riva, S., et al., A new enzymatic route to the synthesis of 12-ketoursodeoxycholic acid, *Biotechnol. Lett.*, 18, 305–308, 1996.
29. Chiari, M., Dell'Orto, N., Mendoza, M., et al., Enzyme reactions in a multicompartment electrolyzer with isoelectronically trapped enzymes, *J. Biochem. Biophys. Methods*, 31, 93–104, 1996.
30. Mashige, F., Kazuhiro, I., and Osuga, T., A simple and sensitive assay of total serum bile acids, *Clin. Chim. Acta*, 70, 79–86, 1976.
31. Mashige, F., Tanaka, N., Maki, A., et al., Direct spectrophotometry of total bile acids in serum, *Clin. Chem.*, 27, 1352–1356, 1981.
32. Styrelius, I., Thore, A., and Bjorkhem, I., Bioluminescent assay for total bile acids in serum with use of bacterial luciferase, *Clin. Chem.*, 29, 1123–1127, 1983.
33. Roda, A., Girotti, S., Carrea, G., et al., Continuous-flow assays with nylon tube-immobilized bioluminescent enzymes, in *Methods in Enzymology*, vol. 137, Mosbach, K., Ed., Academic Press, New York, pp. 161–171, 1988.
34. Klibanov, A.M., Improving enzymes by using them in organic solvents, *Nature*, 409, 241–246, 2001.

35. Carrea, G. and Riva, S., Properties and synthetic applications of enzymes in organic solvents, *Angew. Chem. Int. Ed. Engl.*, 33, 2226–2254, 2000.
36. Zaks, A. and Klibanov, A.M., Enzyme catalyzed processes in organic solvents, *Proc. Natl. Acad. Sci. USA*, 82, 3192–3196, 1985.
37. Therisod, M. and Klibanov, A.M., Facile enzymatic preparation of monoacylated sugars in pyridine, *J. Am. Chem. Soc.*, 108, 5638–5640, 1986.
38. Riva, S., Chopineau, J., Kieboom, A.P.G., et al., Protease-catalyzed regioselective esterification of sugars and related compounds in anhydrous dimethylformamide, *J. Am. Chem. Soc.*, 110, 584–589, 1988.
39. Riva, S. and Klibanov, A.M., Enzymo-chemical regioselective oxidation of steroids without oxidoreductases, *J. Am. Chem. Soc.*, 110, 3291–3295, 1988.
40. Riva, S., Bovara, R., Ottolina, G., et al., Regioselective acylation of bile acids derivatives with *Candida cylindracea* lipase in organic solvents, *J. Org. Chem.*, 54, 3161–3164, 1989.
41. Bertinotti, A., Carrea, G., Ottolina, G., et al., Regioselective esterification of polyhydroxylated steroids by *Candida antarctica* lipase, *Tetrahedron*, 50, 13165–13172, 1994.
42. Cruz, S.M.M., Riva, S., and Sa e Melo, M.L., Regioselective enzymatic acylation of vicinal diols of steroids, *Tetrahedron*, 61, 3065–3073, 2005.
43. Cruz, S.M.M., Riva, S., and Sa e Melo, M.L., Highly selective lipase-mediated discrimination of diastereomeric 5,6-epoxysteroids, *Tetrahedron Asymmetry*, 15, 1173–1179, 2004.
44. Ferraboschi, P., Colombo, D., and Reza-Elahi, S., A practical chemoenzymatic approach to the synthesis of 3-hydroxy metabolites of tibolone, *Tetrahedron Asymmetry*, 13, 2583–2586, 2002.
45. Ferraboschi, P., Colombo, D., and Prestileo, P., A convenient synthesis of oxandrolone through a regioselective *Candida antarctica* lipase-catalyzed transformation, *Tetrahedron Asymmetry*, 14, 2781–2785, 2003.
46. Danieli, B., Lesma, G., Luisetti, M., et al., *Candida antarctica* lipase B catalyzes the regioselective esterification of ecdysteroids at the C-2 OH, *Tetrahedron*, 53, 5855–5862, 1997.
47. Ottolina, G., Carrea, G., and Riva, S., Regioselective enzymatic preparation of hemisuccinates of polyhydroxylated steroids, *Biocatalysis*, 5, 131–136, 1991.
48. Bruttomesso, A.C., Tiscornia, A., and Baldessari, A., Lipase-catalyzed preparation of biologically active esters of dehydroepiandrosterone, *Biocatal. Biotransformation*, 22, 215–220, 2004.
49. Ferraboschi, P., Molatore, A., Verza, E., et al., The first example of lipase-catalyzed resolution of a stereogenic center in steroid side chains by transesterification in organic solvent, *Tetrahedron Asymmetry*, 6, 1551–1554, 1996.
50. Ferraboschi, P., Molatore, A., Verza, E., et al., The first example of lipase-catalyzed resolution of a stereogenic center in steroid side chains by transesterification in organic solvent, *Addendum. Tetrahedron Asymmetry*, 6, 2160, 1996.
51. Ferraboschi, P., Reza-Elahi, S., Verza, E., et al., Lipase-catalyzed resolution of stereogenic centers in steroid side chains by transesterification in organic solvents: the case of a 26-hydroxycholesterol, *Tetrahedron Asymmetry*, 9, 2193–2196, 1998.
52. Ferraboschi, P., Pecora, F., Reza-Elahi, S., et al., Chemoenzymatic syntheses of (25R)- and (25S)-25-hydroxy-27-nor-cholesterol, a steroid bearing a secondary hydroxyl group in the side chain, *Tetrahedron Asymmetry*, 10, 2497–2500, 1999.
53. Santaniello, E., Ferraboschi, P., and Reza-Elahi, S., Lipase-catalyzed regio- and stereoselective acylation of hydroxy groups in steroid side chains, *Mon. Chemie*, 131, 617–622, 2000.
54. Cruz, S.M.M., Sa e Melo, M.L., Parolin, M., et al., The biocatalyzed stereoselective preparation of polycyclic cyanohydrins, *Tetrahedron Asymmetry*, 15, 21–27, 2004.
55. Njar, C.O. and Caspi, E., Enzymatic transesterification of steroid esters in organic solvents, *Tetrahedron Lett.*, 28, 6549–6552, 1987.
56. Baldessari, A., Maier, M.S., and Gros, E.G., Enzymatic deacetylation of steroids bearing labile functions, *Tetrahedron Lett.*, 36, 4349–4352, 1995.
57. Baldessari, A., Bruttomesso, A.C., and Gross, E.G., Lipase-catalyzed regioselective deacylation of androstane derivatives, *Helv. Chim. Acta*, 79, 999–1004, 1996.
58. Bruttomesso, A.C. and Baldessari, A., Lipase-catalysed deacetylation of androstane and pregnane derivatives: influence of ring D substitution, *J. Mol. Cat., B Enzym.*, 29, 149–153, 2004.
59. Secundo, F., Carrea, G., De Amici, M., et al., A combinatorial biocatalysis approach to an array of cholic acid derivatives, *Biotechnol. Bioeng.*, 81, 391–396, 2003.

24 Recent Developments in Enzymatic Acyloin Condensations

Owen Ward and Ajay Singh

CONTENTS

24.1	Introduction	605
24.2	<i>R</i> -PAC Production	606
24.2.1	Reaction Description	606
24.2.2	Strains	607
24.2.3	Biotransformations in Aqueous Media	608
24.2.4	Reaction Specificity	609
24.2.5	Aqueous–Organic Systems	610
24.2.6	Use of Mutants	610
24.2.7	Use of Purified Enzymes	611
24.3	Benzoylformate Decarboxylase	612
24.3.1	The Ligation Reaction	613
24.3.2	Development of Mutants	614
24.4	Phenylpyruvate Decarboxylase	614
24.5	Other Enzymes with Known or Potential Roles in Ligation-Mediated Biotransformations	614
24.5.1	Other α -Ketoacid Decarboxylases	614
24.5.2	Other Thiamine Diphosphate–Dependent Enzymes	615
24.6	Conclusions	616
	References	617

24.1 INTRODUCTION

The production of (*R*)-phenylacetylcarbinol (*R*-PAC), as a precursor intermediate in the synthesis of L-ephedrine, was one of the first industrial biotransformations to be exploited. The reaction is catalyzed by fermenting baker's yeast, *Saccharomyces cerevisiae*, as a side reaction of pyruvate decarboxylase (PDC), a thiamine pyrophosphate (TPP)-linked α -ketoacid decarboxylases. *R*-PAC can be produced by chemical synthesis from cyanohydrins, but biotransformation represents the preferred industrial synthesis route. Other α -ketoacid decarboxylases include benzoylformate decarboxylase, phenylpyruvate decarboxylase, phosphonopyruvate decarboxylase, indole-3-pyruvate decarboxylase, and 3-sulfopyruvate decarboxylase. Bacterial and yeast decarboxylases have been evaluated as biocatalysts not only for the synthesis of acyloin-type compounds but also for the production of hydroxyl ketones [1], α -arylacetate [2], D-amino acids [3], dopamine [4], and organic acids [5]. Some

other TPP-dependent enzymes, which are not α -ketoacid decarboxylases, also have potential roles in bioorganic synthesis.

Decarboxylases have various roles in nature: in the nonoxidative decarboxylation of α - and β -ketoacids and in the synthesis of carbohydrates [6]. Their application as biocatalysts in industrial biotransformation reactions derives from their ability to synthesize enantiopure compounds or precursors with existing or potential applications in the pharmaceutical industry. These enzymes may be used in the form of whole cells or extracted enzymes, in both free and immobilized states. Use of biocatalysts, such as α -ketoacid decarboxylases in chemoorganic synthesis, avoids the problem of isomerization, racemization, epimerization, and rearrangement, typically associated with organic synthesis reactions, as biotransformations are implemented under mild reaction conditions [7]. This chapter discusses recent developments in the applications of enzymes in acyloin-type condensations.

24.2 R-PAC PRODUCTION

24.2.1 REACTION DESCRIPTION

The main reaction (Figure 24.1) in which PDC participates in metabolism is the nonoxidative decarboxylation of α -ketoacids to the corresponding aldehydes [8–10]. The enzyme is activated by its natural substrate, pyruvate. The catalytic mechanism, involving the enzyme-bound reaction intermediate, 2- α -hydroxyethylthiamine pyrophosphate (HETPP), has been extensively investigated [11–13]. The ability of the TPP coenzyme to bind to the carbonyl group and act as an electron sink is what makes decarboxylation of the α -ketoacid possible [14]. Acetaldehyde inhibits the decarboxylation reaction, but has been reported as not

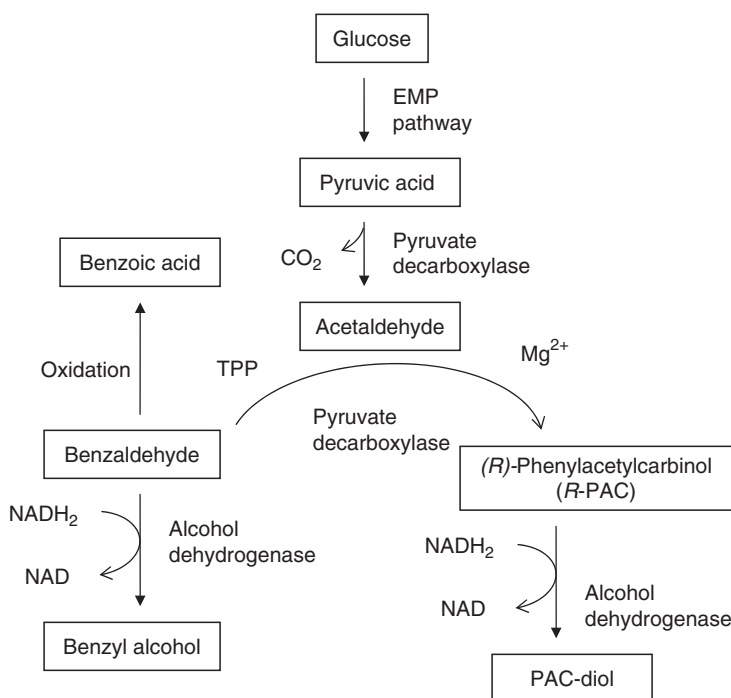


FIGURE 24.1 Pathway for the biosynthesis of (*R*)-phenylacetylcarbinol (*R*-PAC) and associated products.

inactivating the enzyme [9], and the inhibition decreases with increasing chain length of the substrate. PDCs (EC 4.1.1.1) have been characterized from many yeast, bacterial, and plant sources [15,16]. All appear to be made up of subunits consisting of around 560 to 610 amino acids, but with different quaternary structures. The enzyme from baker’s yeast is a tetramer of similar subunits, while the brewer’s yeast enzyme is an $\alpha 2\beta 2$ tetramer [8,17]. The holoenzyme contains as cofactors two to four molecules of TPP and magnesium. The structures of a number of PDCs have been fully elucidated [18–20]. With the exception of PDC from *Zymomonas mobilis*, all enzymes that have been characterized in detail exhibit a sigmoidal plot. Important microbial sources of decarboxylases are shown in Table 24.1.

The “carboligase” side reaction leads to the production of hydroxyl ketones or acyloins [14,21,22] and involves a two-site mechanism of formation of the acyloin compound by PDC. At the first site, pyruvate is decarboxylated to the aldehyde–diphosphatamine complex called “active acetaldehyde.” Active acetaldehyde is irreversibly transferred to the second site, where reversible dissociation of free aldehyde may occur. However, in addition to forming the free aldehyde, PDC also catalyzes the formation of C–C bonds through the acyloin reaction in which free aldehyde competes with a proton for bond formation with the α -carbanion of HETPP. The addition of the C2 unit produces (*R*)-hydroxy ketone [23]. Acyclic and aromatic unsaturated aldehydes have been used in these acyloin condensations in addition to acetaldehyde.

24.2.2 STRAINS

A large number of microbes in addition to *S. cerevisiae* have been shown to produce *R*-PAC by biotransformation. Shukla and Kulkarni [24] noted the capacities of the yeasts *Hansenula*

TABLE 24.1
Microbial Sources of α -Ketoacid Decarboxylases

Enzyme	Microbial Sources		
	Bacteria	Yeast	Filamentous Fungi
Pyruvate decarboxylases (EC 4.1.1.1)	<i>Zymomonas mobilis</i> , <i>Zymobacter palmae</i> , <i>Acetobacter pasteurianus</i> , <i>Sarcina</i> <i>Ventriculi</i>	<i>Candida flarerii</i> , <i>Kluyveromyces marxianus</i> , <i>Saccharomyces cerevisiae</i> , <i>S. carlsbergensis</i> , <i>S. ellipsoideus</i> , <i>S. delbrueckii</i> <i>Zygosaccharomyces rouxii</i> , <i>Hansenula anomala</i> , <i>Brettanomyces vini</i> , <i>Torula utilis</i> , <i>Torulaspora delbrueckii</i> , <i>Schizosaccharomyces pombe</i>	<i>Neurospora crassa</i> , <i>Rhizopus javanicus</i> , <i>Aspergillus niger</i> , <i>A. parasiticus</i> <i>A. nidulans</i> , <i>R. oryzae</i> , <i>Mucor rouxii</i> <i>Fusarium</i> sp., <i>M. circinelloides</i>
Benzoylformate decarboxylases (EC 4.1.1.7)	<i>Pseudomonas putida</i> , <i>P. aeruginosa</i> , <i>Acinetobacter calcoaceticus</i>		
Phenylpyruvate decarboxylases (EC 4.1.1.43)	<i>Achromobacter eurydice</i> , <i>A. calcoaceticus</i> , <i>P. putida</i> , <i>Thauera aromatica</i>	<i>S. cerevisiae</i>	

anomala, *Brettanomyces vini*, *S. carlsbergensis*, *S. ellipsoideus*, *S. delbrueckii*, *Torulaspora delbrueckii*, and *Torula utilis* to produce significant amounts of *R*-PAC. Rosche et al. [25] evaluated 105 yeast strains from 10 genera (*Candida*, *Saccharomyces*, *Nadsonia*, *Kloeckera*, *Cluyveromyces*, *Hansenula*, *Torulopsis*, *Pichia*, *Schizosaccharomyces*, and *Cryptococcus*) and 40 species for *R*-PAC production using a cell-free carboligase assay with benzaldehyde and pyruvate as substrates, and detected activity in all but seven of the strains. Highest activities were found in strains of *Candida utilis*, *C. tropicalis*, and *C. albicans*. In other studies, *Torulopsis glabrata* was identified as a very high *R*-PAC producer [26]. PAC was not formed from benzaldehyde and acetaldehyde by these yeasts. Most of these yeast strains were also found [27] to have carboligase activity and were capable of fermenting glucose. Rosche et al. [28] described a screening program of fungi, covering a broad taxonomic spectrum of Ascomycota, Zygomycota, and Basidiomycota, in which benzaldehyde and pyruvate were transformed into *R*-PAC by all 16 strains tested. Species evaluated included *Rhizopus javanicus*, *R. oryzae*, *Aspergillus oryzae*, *A. tamari*, *Neurospora crassa*, *Polyporus eucalyptorum*, *Fusarium lateritium*, another *Fusarium* sp., *Monilia sitophila*, *Paecilomyces lilacus*, and *Mucor rouxii*. In 12 of the fungal strains tested, enantiomeric excesses (ees) of *R*-PAC were $\geq 90\%$, similar to the values that were obtained with strains of *C. utilis* and *S. cerevisiae* which were tested in the same experiment. Highest *R*-PAC yields were observed with *F. javanicus* and another *Fusarium* sp.

Silk and Macaulay [29] observed that many metabolites, including PAC and PAC derivatives, are produced by culture extracts of *Bjerkandera adjusta*, incubated with phenylalanine, with or without cosubstrates such as glycerol, pyruvate, or glucose.

Some species such as *Saccharomyces*, *Candida*, *Hansenula*, *Rhizopus*, *Aspergillus*, *Fusarium*, *Monilia*, *Paecilomyces*, and *Mucor* can synthesize *R*-PAC in the range of 4.5 to 20 g/L [28,30,31].

In contrast to the carboligase activity exhibited by yeasts and fungi, which cannot utilize acetaldehyde as substrate in place of pyruvate, PDCs from the bacteria *Z. mobilis* and *Zymobacter palmae* can use acetaldehyde as substrate [32]. This has significance commercially, as acetaldehyde is considerably less expensive than pyruvate [33].

24.2.3 BIOTRANSFORMATIONS IN AQUEOUS MEDIA

The conventional commercial production of *R*-PAC consists of a yeast growth stage facilitating biomass production, accumulation of an intracellular pyruvate pool, and induction of PDC, followed by a benzaldehyde-fed whole-cell biotransformation stage with benzaldehyde concentration maintained below inhibiting levels. With *S. cerevisiae*, optimal concentration of benzaldehyde for *R*-PAC production was indicated to be 10 mM [22]. The yeast whole-cell biotransformation process is limited by the denaturing effects of benzaldehyde on the biocatalytic system, by insufficient accumulation of pyruvate, and by reduction in *R*-PAC yields as a result of diversion of substrates into by-products, mediated by other yeast enzymes [34].

Increased benzaldehyde concentrations decreased yeast cell viability [35] and caused a cessation in *R*-PAC production. While cells grew at a reduced rate of 0.5 g/L benzaldehyde, concentrations of 1 to 2 g/L completely inhibited growth and 3 g/L reduced cell viability, indicating that benzaldehyde concentrations for *R*-PAC production should be maintained at or below 1 g/L. Higher benzaldehyde concentrations were found to damage yeast cell permeability, with associated losses of cofactors, rather than by inactivating PDC activity. Wang et al. [36] exploited a benzaldehyde feed strategy to accumulate *R*-PAC to a concentration of 22 g/L. Other strategies involved development of aldehyde-resistant mutants. Addition of β -cyclodextrin caused entrapment and controlled release of benzaldehyde, reduction of toxicity toward the cells, and increased *R*-PAC yield [37]. Yields of more than 22 g/L *R*-PAC were

obtained using a wild-type strain of *C. utilis* through optimal control of metabolism by microprocessor-control of respiratory quotient to enhance pyruvate production and induction of PDC activity [38].

Quantitative conversion of benzaldehyde to *R*-PAC is never achieved due to the formation of by-products. Although a variety of by-products have been observed including acetyl benzoyl, *trans*-cinnamaldehyde, 2-hydroxy-1,1-phenylpropane, and benzyl alcohol, only the latter is produced in amounts substantial enough to influence the efficiency of *R*-PAC production [39–41]. Benzyl alcohol is formed from benzaldehyde in a reaction mediated by yeast alcohol dehydrogenase [42,43]. Ose and Hironaka [44] demonstrated that addition of acetaldehyde to the biotransformation increased and reduced formation of *R*-PAC and benzyl alcohol, respectively, and this may be attributed to the observation that alcohol dehydrogenase is more susceptible to denaturation by acetaldehyde than is PDC [35].

Miyata [26] addressed the problem of pyruvate limitation using a strain of *T. glabrata* and succeeded in producing a conversion to *R*-PAC of 70%, based on benzaldehyde, and volumetric yields of up to 30 g/L *R*-PAC.

Yeast cell immobilization reduced the toxic effect of benzaldehyde likely due to diffusion limitations within the immobilizing matrix [45–47]. By increasing benzaldehyde concentrations in the range of 2 to 6 g/L in an immobilized system, *R*-PAC concentrations were increased up to 7.5-fold the amount observed in free-cell controls. A high *R*-PAC concentration was produced by *S. cerevisiae* cells immobilized in an ENT-4000 matrix (containing polyethylene glycol). The nature of the support material also affected the ratio of product *R*-PAC to by-product benzyl alcohol [48]. Mahmoud et al. [47] exploited immobilized yeast cell systems to prolong semicontinuous transformation reactions and to reuse the cells through multiple [7–9] cycles. Shukla and Kulkarni [49] used *T. delbrueckii*, immobilized in alginate gels, for *R*-PAC synthesis and achieved superior yields with barium alginate compared with calcium alginate.

However, immobilized cell systems also limit rates of transformation, with diffusion limitations of substrates into the matrix and products from the matrix likely being the dominant causative factor responsible for the low transformation rates.

24.2.4 REACTION SPECIFICITY

PDC has been shown to be able to decarboxylate not only pyruvate but also hydroxyl pyruvate, acetaldehyde, aliphatic α -ketoacids, *p*-substituted α -ketoacids, and a number of higher homologs of pyruvate up to 2-oxohexanoate [17,50]. *S. cerevisiae* was found to be able to produce acyloin condensations of pyruvate by replacing benzaldehyde with substituted aromatic aldehydes, although acyloin yields were lower than those observed for benzaldehyde as substrate [42]. Aldehydes with substituents in the *ortho*-position were demonstrated consistently to be poor substrates for carbinol production. Aromatic aldehydes with $-\text{CH}_3$, $-\text{CF}_3$, and $-\text{Cl}$ substituents located in the *para*-position produced higher carbinol yields than their *meta*-counterparts.

Acetohydroxyacid synthase, whose normal physiological function is the synthesis of (*S*)-acetohydroxyacids from pyruvate and a second ketoacid, also has the ability to catalyze chiral synthesis of *R*-PAC from pyruvate and benzaldehyde [51]. Some *Escherichia coli* acetohydroxyacid synthase isoenzymes, especially isoenzyme I, have advantages over PDC in this biotransformation in that high conversion yields of *R*-PAC are obtained and there is negligible acetaldehyde formation. Acetohydroxyacid synthase appears to be able to convert pyruvate and a range of substituted benzaldehydes and other aromatic aldehydes into the corresponding PAC analogs. In addition, pyridine, thiophene, furan, and naphthyl aldehydes are acceptable substrates in the biotransformation.

Culture extracts of *B. adjusta*, produce PAC from phenylalanine, with and without cosubstrate, and generate PACs substituted differently at the aromatic and/or aliphatic side depending on the nature of the cosubstrate used in the reaction [29]. When PAC was incubated as substrate with the culture extracts, eight different biotransformation products were observed, indicating the potential to use enzymes from this strain to produce substituted PAC products. Many of the reactions described are stereoselective.

24.2.5 AQUEOUS–ORGANIC SYSTEMS

Nikolova and Ward [52,53] investigated *R*-PAC production by yeast in a two-phase aqueous–organic biotransformation system. When the effect of moisture content on *R*-PAC production by yeast cells, immobilized to celite, was investigated using hexane as organic solvent, a maximum biotransformation activity was observed at a moisture level of 10%. Better transformation activities were observed in hydrophobic solvents like hexane and hexadecane compared to more hydrophilic solvents like chloroform, ethyl-, and butyl acetate. These latter solvents also caused puncturing of the yeast cells. Laurence et al. [54] used lactic acid as cosubstrate and a pyruvate precursor with benzaldehyde in yeast *R*-PAC biotransformations in petroleum spirit, but rates of transformation were inferior to those obtained with pyruvate.

With monophasic biotransformation systems, containing water-miscible organic solvents, for *R*-PAC production using aldehyde-resistant yeast mutants, *R*-PAC concentrations of up to 15 g/L have been observed, with associated reduced production of benzyl alcohol [36]. When the yeast-mediated condensation of benzaldehyde and pyruvate was carried out in a petroleum spirit solvent system, addition of a small amount of ethanol (0.5%) was found to inhibit benzyl alcohol production and to increase *R*-PAC yield [55]. Solvents such as ethanol, other monohydric solvents, and long- and short-chain polyols, such as ethylene glycol and glycerol, appeared to stimulate *R*-PAC production by immobilized yeast cell mass [56].

24.2.6 USE OF MUTANTS

Mutant strains of *S. cerevisiae* and *C. flarerii*, resistant to acetaldehyde and *R*-PAC, produced more of the condensation product than the wild-type parent strains, but not more than 10 g/L [57]. Mutant strains of *S. cerevisiae*, lacking some or all of the alcohol dehydrogenase isoenzymes I, II, and III, manifested similar rates of conversion of benzaldehyde to the undesirable by-product, benzyl alcohol [58].

Site-directed mutagenesis and molecular approaches have recently been used to better understand enzyme structure–function relationships of biocatalytic effects in the PAC biotransformation. Site-directed mutagenesis, replacing a Trp392 with Ala, enhanced the typically low carbonylase activity of *Z. mobilis* PDC, but enzyme conformational stability was reduced [59]. Replacement of Trp with Ile or Met increased biotransformation activity (five to six times) as well as conformational stability. A number of other hydrophobic amino acid site-specific mutations in or around the active site (Leu112, Ile472, and Ile476) of the *Z. mobilis* PDC have been probed with respect to their effects on relative decarboxylation/carbonylation activity, substrate specificity, and enantiospecificity [15].

Acetohydroxyacid synthase from *E. coli* can catalyze the stereospecific conversion of pyruvate and benzaldehyde to *R*-PAC. Studies on the effects of mutation of four different amino acids at the active site on biotransformation reaction rates and substrate specificity of the ligation reaction demonstrated the crucial role of Arg276 in stabilizing enzyme–first substrate transition states for ligation of the incoming second substrate [60]. These studies demonstrated the potential to engineer this particular enzyme to enable it to catalyze novel reactions of possible industrial utility.

24.2.7 USE OF PURIFIED ENZYMES

Because use of purified PDC enzymes avoids the problem of by-product formation due to the presence of alcohol dehydrogenases, purified PDCs from *S. cerevisiae*, *C. utilis*, and *Z. mobilis* have been evaluated for synthesis of *R*-PAC (Figure 24.2) [56,61]. Both *C. utilis* and *S. cerevisiae* enzymes were effective in producing *R*-PAC and were stable only at low temperatures. Shin and Rogers [61] used a partially purified PDC from *C. utilis* to achieve final *R*-PAC concentrations of 28 g/L. In contrast to *C. utilis*, the *Z. mobilis* PDC exhibited better stability and high catalytic activity at room temperature. However, this enzyme is disadvantageous in that it has low affinity for benzaldehyde and exhibits considerable substrate inhibitory effects.

Iwan et al. [62] exploited the high stability toward acetaldehyde of mutated PDC enzymes, PDCW392I and PDCW392, from *Z. mobilis* mutants, in continuous *R*-PAC production systems using enzyme-membrane bioreactors. With pyruvate (90 mM) and benzaldehyde (30 mM) as substrates, *R*-PAC was produced in a space-time yield of 27.4 g/L/d using purified PDCW392I. With acetaldehyde (50 mM) and benzaldehyde (50 mM) as substrates, a space-time yield of 81 g/L/d was obtained with PDCW392M. This approach also overcomes the problem of by-product formation in current fermentation processes [63].

It was noted earlier that high concentrations of *R*-PAC were obtained in fungal screening studies when cell-free extracts were used as biocatalyst in the biotransformation reaction [28]. Indeed, much lower PAC concentrations (19 mM, 2.9 g/L) were obtained in biotransformations with fermenting mycelia, and this appeared to be due to rapid reduction of the benzaldehyde to the by-product, benzyl alcohol. Using initial substrate concentrations of 100 mM benzaldehyde and 150 mM pyruvate, concentrations of 78 to 84 mM PAC (11.7 to 12.6 g/L) were achieved in reactions with cell-free extracts of *R. javanicus* and *Fusarium* sp. in 20 h incubations. Under the same conditions extracts of *S. cerevisiae* and *C. utilis* produced

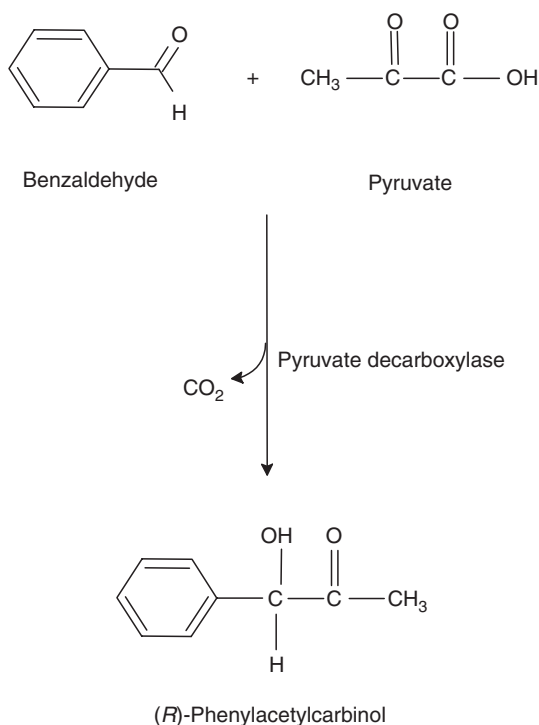


FIGURE 24.2 Enzymatic synthesis of (*R*)-phenylacetylcarbinol by pyruvate decarboxylase (PDC).

11.3 g/L PAC. Under optimized conditions a partially purified PDC from *C. utilis* produced 14.6 g PAC/L from the same substrate concentrations [61].

Rosche et al. [28] reported significant increases in the production of *R*-PAC with the use of enzyme biotransformations in place of whole cells, noting that limitations on use of whole cells arise as a result of benzaldehyde toxicity, insufficient accumulation of pyruvate, and reduction in product yield through production of benzyl alcohol. Pyruvate and benzaldehyde were used as substrates in these biotransformations. Benzaldehyde was present in the form of an emulsion. Reduction in magnesium concentration from 20 to 0.5 mM and implementation of the biotransformation at 6°C resulted in high accumulation of PAC by PDC from *R. javanicus* and *C. utilis*. Lowering the temperature from 23 to 6°C decreased initial reaction rates but increased final *R*-PAC concentrations [28]. Loss of pyruvate has been attributed to temperature and concentration-related impacts of magnesium [64]. The initial optimum pH was 6.5 and the tendency for the pH to rise during the reaction due to proton uptake was countered through judicious buffer selection and use. The *C. utilis* enzyme formed less by-product from pyruvate and was more stable under the biotransformation conditions. Free PDC from yeast and from the fungus, *R. javanicus*, produced concentrations of *R*-PAC in excess of 50 g/L with no formation of benzyl alcohol by-product.

The emulsion process using *C. utilis* PDC was limited by inactivation of the enzyme by benzaldehyde, *R*-PAC, and the by-product acetoin [28]. Even higher concentrations of product (>100 g/L) were achieved in two-phase aqueous–organic systems, containing octanol and nonanol designed to partition the enzyme-inactivating substrate, benzaldehyde, into the organic phase. The product *R*-PAC was also substantially partitioned into the organic phase. In a rapidly stirred two-phased system, buffered at pH 6.5 and incubated at 4°C, containing 160 g/L benzaldehyde and 123 g/L pyruvate, levels of benzaldehyde in the aqueous medium were less than 50 mM. After 49 h, when all pyruvate was used up, final *R*-PAC concentration was 140 g/L, representing a 91% molar conversion from benzaldehyde. In a similar system, not rapidly stirred so as to maintain phase separation, a molar conversion of 93% benzaldehyde to *R*-PAC was achieved but in a more prolonged incubation phase of 395 h. In both the rapidly stirred and phase-separated system ee of *R*-PAC was >99%.

These studies indicated how substantial improvements can be achieved in the *R*-PAC biotransformation: by using isolated enzymes instead of whole cells, thereby eliminating problems of pyruvate limitation and alcohol dehydrogenase-mediated benzyl alcohol formation; and by employing a two-phase aqueous–organic system to partition the substrate benzaldehyde predominantly in the organic phase, thereby reducing its inactivating effect on PDC located in the aqueous phase.

Leksawasdi et al. [65] implemented initial rate and biotransformation investigations in mathematical modeling studies on *C. utilis* PDC-mediated production of *R*-PAC from pyruvate and benzaldehyde. The model was validated as being capable of predicting time courses for substrates, *R*-PAC, by-products acetaldehyde and acetoin, and enzyme activity level.

Rosche et al. [66] screened organic solvents for use in the biphasic biotransformation of acetaldehyde and benzaldehyde to *R*-PAC by PDC isolated from *Z. mobilis* PDCW392M. Best PAC formation was observed with alcohols, in particular 1-pentanol, 1-hexanol, and isobutanol, which, in contrast to other solvents, produced lower concentrations of toxic acetaldehyde substrate in the aqueous phase.

24.3 BENZOYLFORMATE DECARBOXYLASE

Benzoylformate decarboxylase (EC 4.1.1.7) catalyses conversion of benzoylformate to benzaldehyde [67–69], and is an integral part of the mandelic acid pathway for catabolism of aromatic compounds by different *Pseudomonas* and *Acinetobacter* sp. The purified enzymes

from *Acinetobacter calcoaceticus* [67] and *P. putida* [70,71] have tetrameric structures like PDC and are likewise TPP- and Mg-dependent enzymes. Benzoylformate decarboxylase has higher substrate specificity than PDC, using only benzoylformate and *para*-substituted benzoylformates as substrates [70–72].

24.3.1 THE LIGATION REACTION

Wilcocks et al. [73] demonstrated the capacity of cells of *P. putida* containing benzoylformate decarboxylase to form the acyloin compound (*S*)-2-hydroxypropiophenone or (*R*)-benzoin, when incubated with benzoylformate and acetaldehyde [21]. Figure 24.3 shows a benzoylformate decarboxylase catalyzed reaction. Cell extract or purified enzyme was also able to carry out the same biotransformation. Accumulation of benzyl alcohol was observed in reactions catalyzed by whole cells or cell extracts. The product was formed with a high degree of enantiospecificity, having an ee of 91 to 92% as 2-(*S*)-hydroxypropiophenone [74]. Under optimized conditions, in the presence of 100 mM benzoylformate and 1600 mM acetaldehyde, 61.76 mM acyloin product was produced in 1 h. Stereoselectivity of the reaction was improved by the use of *A. calcoaceticus* cells instead of *P. putida*, where optical purity of the (*S*)-enantiomer was >98% [75–77]. Optimization of reaction conditions resulted in the achievement of extremely high productivities, namely 6.95 g product/L/h.

Demir et al. [78] described the first general synthesis of benzoin and substituted benzoin, mediated by benzoylformate decarboxylase. By increasing the benzaldehyde concentration or decreasing the acetaldehyde concentration in the reaction mixture, (*R*)-benzoin formation was observed. Optimization of biotransformation conditions with respect to time, enzyme, cofactor, and medium produced a yield of 70% of (*R*)-benzoin, having a >99% ee. Addition of dimethylsulfoxide had no effect on ee but increased biotransformation reaction rate. Park and Jung [79] found that encapsulation of whole cells of *P. putida* in calcium alginate liquid core capsules resulted in the biotransformation of benzaldehyde and acetaldehyde to 2-hydroxypropiophenone, proceeding without the associated production of the undesirable

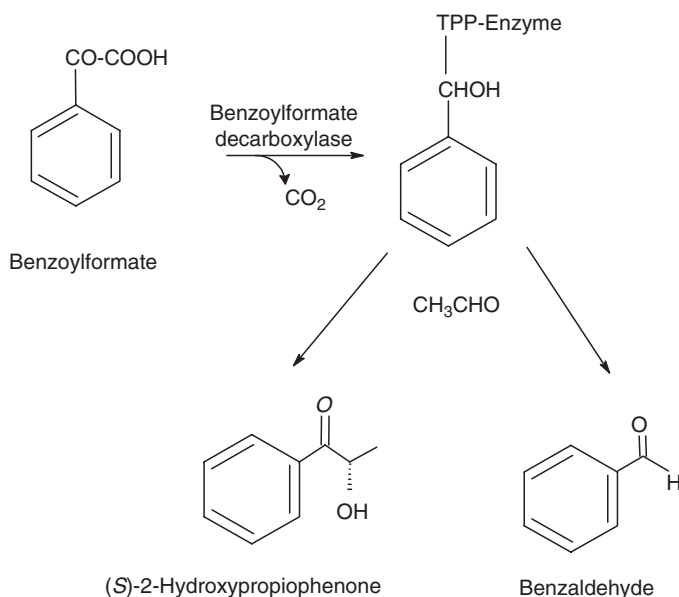


FIGURE 24.3 Synthesis of (*S*)-2-hydroxypropiophenone by benzoylformate decarboxylase.

by-product, benzyl alcohol. In addition, the cells retained their biotransformation activity with minimum loss by recycling many times.

24.3.2 DEVELOPMENT OF MUTANTS

The gene encoding the *P. putida* enzyme and the crystal structure of the protein have been characterized [80,81]. Lingen et al. [82] used error-prone polymerase chain reaction (PCR) to develop benzoylformate decarboxylase mutant enzymes with altered substrate specificities. Mutant enzymes L476Q and M365L-L461S accepted *ortho*-substituted benzaldehyde derivatives such as 2-chloro-benzaldehyde or 2-bromo-benzaldehyde as donor substrates, leading to production of 2-hydroxyketones, and produced enantiopure (*S*)-2-hydroxy-1-(2-methylphenyl)propan-1-one with excellent yields. The wild-type enzyme shows very poor catalytic activity toward this reaction. In aqueous buffer benzoylformate decarboxylase L476Q also exhibited a fivefold increase in carboligase activity compared to the wild-type enzyme producing (*S*)-2-hydroxy-1-phenyl-propanone with a high ee. From modeling investigations of the structure of the wild-type enzyme, it has been postulated that reduction of the side chain size at L461 can alter the substrate specificity of the enzyme by facilitating accommodation and binding the enlarged *ortho*-substituted donor substrates to the thiamine diphosphate.

24.4 PHENYLPYRUVATE DECARBOXYLASE

Phenylpyruvate decarboxylase (EC 4.1.1.43), another TPP-dependent α -ketoacid decarboxylase, has been observed in *Acinetobacter auridice*, *A. calcoaceticus* [68,83], *Thauera aromatica* [84], and *S. cerevisiae* [85], but there are few studies on the properties of this enzyme. Phenylpyruvate decarboxylase activity appears to be induced when cells are grown on substrates like mandelate [67,86], phenylalanine, or tryptophan [84]. The enzyme participates in conversion of phenylpyruvate to phenylacetate, through phenylacetaldehyde. Like PDC and benzoylformate decarboxylase, this enzyme is also a tetramer, and has four identical subunits. The substrate specificity of phenylpyruvate decarboxylase is broader than that of benzoylformate decarboxylase, with compounds like indolepyruvate and α -ketoacids, having more than six carbon atoms in a straight chain, acting as substrates. Phenylpyruvate decarboxylase activity was present in *S. cerevisiae* cultures grown with phenylalanine as sole nitrogen source, and the genes for this enzyme have been identified and characterized [85]. Phenylpyruvate decarboxylase is presumed to be important in the production of fusel oils by yeast, one component of which is phenylethanol [87].

Asymmetric acyloin condensations catalyzed by phenylpyruvate decarboxylase have also been reported [88]. *Achromobacter eurydice*, *Pseudomonas aromatica*, and *P. putida* cells grown on L-phenylalanine catalyzed the acyloin condensation of phenylpyruvate and acetaldehyde to produce 3-hydroxy-1-phenyl-2-butanone (Figure 24.4) with high degrees of enantioselectivity.

24.5 OTHER ENZYMES WITH KNOWN OR POTENTIAL ROLES IN LIGATION-MEDIATED BIOTRANSFORMATIONS

24.5.1 OTHER α -KETOACID DECARBOXYLASES

Three other α -ketoacid decarboxylases—phosphonopyruvate decarboxylase, indole-3-pyruvate decarboxylase, and 3-sulfo-pyruvate decarboxylase—have been characterized. Phosphonopyruvate decarboxylase [89] participates in the biosynthetic pathways for production of

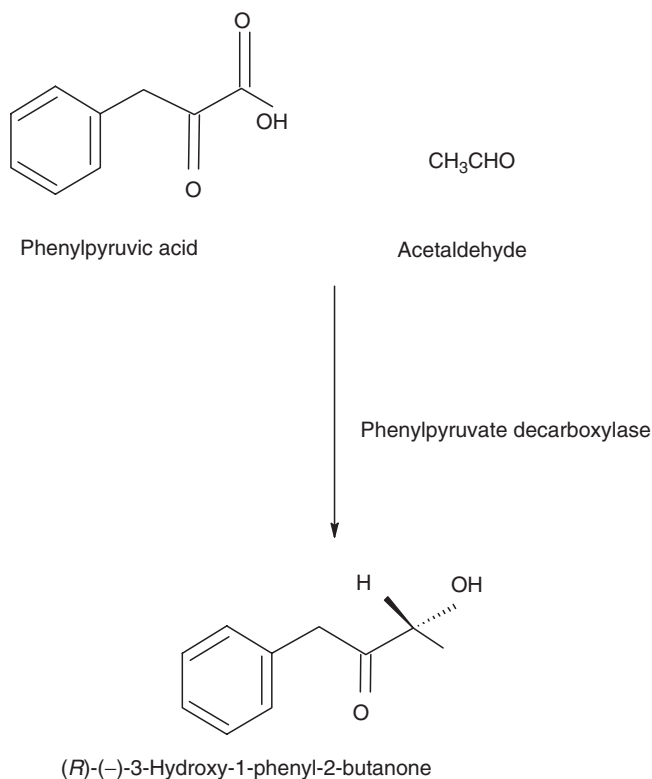


FIGURE 24.4 Asymmetric acyloin condensation catalyzed by phenylpyruvate decarboxylase.

bialaphos, fosfomycin, phosphinothricin, and tripeptide antibiotics. This enzyme is produced by *Bacteroides fragilis* and certain *Streptomyces* spp. Indole-3-pyruvate decarboxylase, involved in tryptophan catabolism of *Enterobacter cloacae* and many other bacteria [90,91], is a tetrameric enzyme with four active sites and has structural homology with the above decarboxylases. *Archea* spp. produce 3-sulfoypyruvate decarboxylases that participate in coenzyme M biosynthesis [92].

Sequence similarities among PDCs, phenylpyruvate decarboxylase, and indole-3-pyruvate decarboxylase are greater than 50%. Benzoylformate decarboxylase shows less than 30% sequence similarity with the above group [93]. Phosphonopyruvate decarboxylase and 3-sulfoypyruvate decarboxylases appear to be more distantly related both in terms of structure and amino acid sequence. Structural and mutagenesis investigations have established that differences in the amino acid sequences near the active sites of α -ketoacid decarboxylases strongly influence substrate selectivity and enantiospecificity of this group of enzymes [94].

24.5.2 OTHER THIAMINE DIPHOSPHATE-DEPENDENT ENZYMES

Apart from the α -ketoacid decarboxylases, a variety of other TPP-dependent enzymes have the potential to participate in ligation reactions, because they also produce active aldehyde intermediates. Acetohydroxyacid synthase from *E. coli* [95] and acetolactate synthase [96] catalyze decarboxylation of pyruvate, followed by ligation of the active acetaldehyde to the C2 of another α -ketoacid. As indicated above, acetohydroxyacid synthase I from *E. coli* was also shown to catalyze *R*-PAC synthesis [97]. Benzaldehyde lyase catalyzes ligation of aldehydes to chiral 2-hydroxyketones and may be exploited for synthesis of various 2-hydroxyketones

[98,99]. A number of other enzymes with potential applications in C–C ligation reactions in bioorganic synthesis and also some enzymes participating in C–N ligation reactions are described by Pohl et al. [100].

24.6 CONCLUSIONS

Recent developments have substantially advanced our scientific knowledge of α -ketoacid decarboxylases and, indeed, of other TPP-requiring enzymes [100,101]. Extensive screening programs to find *R*-PAC producers have shown a widespread presence of ligase activity capable of synthesizing *R*-PAC from pyruvate and benzaldehyde among fungi and yeasts, and have resulted in the identification of some strains with very high biotransformation activity. While carboligase activity of fungi and yeasts cannot use acetaldehyde as substrate instead of pyruvate, PDCs from bacteria such as *Z. mobilis* and *Zymobacter* can use the less expensive acetaldehyde substrate.

While whole-cell yeast biotransformations have been manipulated to produce up to 20 g/L *R*-PAC, the biotransformation efficiency is limited by the availability of pyruvate for the reaction, by toxic effects of benzaldehyde and *R*-PAC, and by participation of other cellular enzymes in the production of by-products. While whole-cell immobilization approaches could be exploited to prolong the biotransformation or to reduce benzaldehyde toxicity to manipulate *R*-PAC/by-product ratios, rates and extents of transformation were typically limited by diffusion of reactants through the immobilization matrix. Mutation strategies have been used with limited success to make yeast strains more resistant to aldehyde, and ADH-defective mutants still form benzyl alcohol by-product from benzaldehyde. Whole-cell biotransformations in aqueous–organic reaction media could be manipulated to inhibit by-product formation but such systems did not improve biotransformation rates observed in aqueous media.

Purified PDCs have been used to overcome the limitations outlined above for whole-cell *R*-PAC biotransformations. Reduction of incubation temperature from 23 to 4°C minimized losses of pyruvate through nonbiological side reactions, and adequate buffering of the reaction countered the tendency for pH rises due to proton uptake. Use of a continuous enzyme–membrane reactor in the *Zymomonas*-acetaldehyde–benzaldehyde biotransformation produced a space–time yield of 81 g/L/d and was free of undesirable by-products. Incorporation of nonmiscible organic solvents, notably octanol, in the reaction substantially partitioned benzaldehyde and *R*-PAC into the octanol, thereby minimizing the denaturing effects of these reactants on the enzyme. Optimizing these various parameters resulted in production of final concentrations of 100 to 140 g/L *R*-PAC, representing molar conversions of >90% based on benzaldehyde and a product ee of 99%.

The capacities of benzoylformate decarboxylase and phenylpyruvate decarboxylase to carry out efficient and enantiospecific carboligase biotransformations have also been described, and it is assumed that these systems would be likewise amenable to the kinds of reaction optimization strategies that have been applied to PDC.

In the past few years, applications of mutagenesis and molecular methods, combined with advances in our ability to analyze and characterize the structures of these enzymes, have allowed researchers to understand the key structure–functional relationships that are important in their biocatalytic reactions. These approaches have been successfully exploited to understand the roles of amino acids near the active site of PDC and benzoylformate decarboxylase with respect to relative decarboxylation/ligation activity, substrate- and enantiospecificity, and to generate mutants with improved activity and conformational stability. These studies demonstrate how improved biocatalysts may be developed for some of the more established biotransformations, resulting in higher volumetric productivities and yields of desired product.

A number of other α -ketoacid decarboxylases and other thiamine diphosphate-dependent enzymes having potential to participate in ligation reactions have been identified. It is clear that many new enzymes from this family will be discovered and/or engineered in the coming years. In addition, the interesting capacity of *B. adjusta* to produce PAC from phenylalanine and to transform PAC substrates into a variety of PAC-substituted analogs has been noted. These various developments will undoubtedly lead to a diversity of new biocatalytic opportunities for bioorganic synthesis.

REFERENCES

1. Iding, H., Siegert, P., Mesch, K., and Pohl, M., Application of alpha-keto acid decarboxylases in biotransformations, *Biochim. Biophys. Acta*, 1385, 307–322, 1998.
2. Ohta, H. and Sugai, T., Enzyme-mediated decarboxylation reactions in organic synthesis, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, pp. 487–526.
3. Yagasaki, M. and Ozaki, A., Industrial biotransformations for the production of amino acids, *J. Mol. Catal., B Enzym.*, 4, 1–11, 1998.
4. Lee, S.-G., Hong, S.-P., and Sung, M.-H., Development of an enzymatic system for production of dopamine from catechol, pyruvate and ammonia, *Enzyme Microb. Technol.*, 25, 298–302, 1999.
5. Abelyan, V.A. and Abelyan, L.A., Production of L-lactic acid and L-malic acid by using intact and immobilized cells of *Lactobacillus casei*, *Appl. Biochem. Microbiol.*, 34, 578–580, 1998.
6. Schulze, B. and Wubbolts, M.G., Biocatalysis for industrial production of fine chemicals, *Curr. Opin. Biotechnol.*, 10, 609–615, 1999.
7. Ward, O.P. and Singh, A., Enzymatic asymmetric synthesis by decarboxylases, *Curr. Opin. Biotechnol.*, 11, 520–526, 2000.
8. Huebner, G., Koenig, S., Schellenberger, A., and Koch, M.H.J., An x-ray solution scattering study of the cofactor and activator induced structural changes in yeast pyruvate decarboxylase (PDC), *FEBS Lett.*, 266, 17, 1990.
9. Juni, E., Evidence for a two-site mechanism for decarboxylation of alpha-carboxylase, *J. Biol. Chem.*, 236, 2302–2308, 1961.
10. Ulrich, J., Wittorf, J.H., and Gubber, C.J., Molecular weight and coenzyme content of pyruvate decarboxylase from brewer's yeast, *Biochim. Biophys. Acta*, 113, 595–604, 1966.
11. Utter, M.F.M., Non-oxidative carboxylation and decarboxylation, in *The Enzymes*, vol. 5, Boyer, P.D., Lardy, H., and Myrbak, K., Eds., Academic Press, New York, 1961, p. 319.
12. Schellenberger, A., Struktur und wirkungsmechanismus des aktiven zentrums der hefe-pyruvate decarboxylase, *Angew. Chem. Int. Ed. Engl.*, 79, 1050–1061, 1967.
13. Ulrich, J., Structure–function relationship in pyruvate decarboxylases of yeast and wheat germ, *Ann. N.Y. Acad. Sci.*, 378, 287–305, 1982.
14. Kern, D., Kern, G., Neef, H., Tittman, K., Killanberg-Jabs, M., Wikner, C., Schneider, G., and Hubner, G., How thiamin diphosphate is activated in enzymes, *Science*, 275, 67–70, 1997.
15. Pohl, M., Siegert, P., Mesch, K., Bruhn, H., and Grotzinger, J., Active site mutants of pyruvate decarboxylase from *Zymomonas mobilis*—a site-directed mutagenesis study of Lii2, I472, I476, E473 and N482, *Eur. J. Biochem.*, 257, 538–546, 1998.
16. Candy, J.M. and Duggleby, R.G., Structure and properties of pyruvate decarboxylase and site-directed mutagenesis of the *Zymomonas mobilis* enzyme, *Biochim. Biophys. Acta*, 1385, 323–338, 1998.
17. Schomburg, D. and Salzmann, N., Lyases. Pyruvate decarboxylase, in *Enzyme Handbook*, vol. 1, Springer-Verlag, Berlin, 1990, p. 1.
18. Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B.C., and Jordan, F., Catalytic centers in the thiamin diphosphate dependent enzyme pyruvate decarboxylase at 2.4-Å resolution, 1993.
19. Arjunan, P., Umland, T., Dyda, F., Swaminathan, S., Furey, W., Sax, M., Farrenkopf, B., Gao, Y., Zhang, D., and Jordan, F., Crystal structure of the thiamine diphosphate-dependent enzyme pyruvate decarboxylase from the yeast *Saccharomyces cerevisiae* at 2.3 angstrom resolution, *J. Mol. Biol.*, 256, 590–600, 1966.

20. Konig, S., Subunit structure, function and organization of pyruvate decarboxylases from various organisms, *Biochim. Biophys. Acta*, 1385, 271–286, 1998.
21. Sprenger, G.A. and Pohl, M., Synthetic potential of thiamin diphosphate-dependent enzymes, *J. Mol. Catal. B Enzym.*, 6, 145–159, 1999.
22. Tripathi, C.M., Agarwal, S.C., and Basu, S.K., Production of L-phenylacetylcarbinol by fermentation, *J. Ferment Bioeng.*, 84, 487–492, 1997.
23. Csuk, R. and Glanzer, B.I., Baker's yeast mediated transformations in organic chemistry, *Chem. Rev.*, 91, 49, 1991.
24. Shukla, V.B. and Kulkarni, P.R., L-Phenylacetylcarbinol (L-PAC) biosynthesis and industrial applications, *World J. Microbiol. Biotechnol.*, 16, 499–506, 2000.
25. Rosche, B., Breuer, M., Hauer, B., and Rogers, P.L., Screening of yeast for enzymatic (R)-phenylacetylcarbinol production, *Biotechnol. Lett.*, 28, 841–845, 2003.
26. Miyata, R., Igarashi, K., and Yonehara, T., Production of pyruvic acid, Jap. Patent 2,000,078,996, 2000.
27. Barnett, J.A., Payne, R.W., and Yarrow, D., *Yeasts: Characteristics and Identification*, 3rd ed., Cambridge University Press, Cambridge, 2000.
28. Rosche, B., Sandford, V., and Breuer, M., Biotransformation of benzaldehyde into (R)-phenylacetylcarbinol by filamentous fungi or their extracts, *Appl. Microbiol. Biotechnol.*, 57, 309–315, 2001.
29. Silk, P.J. and Macauley, J.B., Stereoselective biosynthesis of chloroarylpropane diols by the basidiomycete *Bjerkandera adusta*: exploring the roles of amino acids, pyruvate, glycerol and phenylacetylcarbinol, *FEMS Microbiol. Lett.*, 228, 11–19, 2003.
30. Baev, M.V. and Ward, O.P., Decarboxylases in stereoselective catalysis, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, pp. 267–287.
31. Oliver, A.L., Anderson, F.A., and Roddick, F.A., Factors affecting the production of L-phenylacetylcarbinol by yeast: a case study, *Adv. Microb. Physiol.*, 41, 1–45, 1999.
32. Breuer, M., Hauer, B., and Mesch, L., Method for producing enantiomer-free phenylacetylcarbinols from acetaldehyde and benzaldehyde in the presence of pyruvate decarboxylase from *Zymomonas*, Ger. Patent 19,736,104 A1, 1997.
33. Hauer, B., Breuer, M., and Rogers, P., Process for production of R-phenylacetylcarbinol by an enzymatic process in a two-phase system, Ger. Patent Application, DE 10,142,574 (WO 03/020942), 2001.
34. Rosche, B., Sandford, V., Breuer, M., Hauer, B., and Rogers, P.L., Enhanced production of R-phenylacetylcarbinol (R-PAC) through enzymatic biotransformation, *J. Mol. Catal. B Enzym.*, 19–20, 109–115, 2002.
35. Long, A. and Ward, O.P., Biotransformation of benzaldehyde by *Saccharomyces cerevisiae*: Characterization of the fermentation and toxicity effects of substrates and products, *Biotechnol. Bioeng.*, 34, 933–941, 1989.
36. Wang, B., Shin, H.S., and Rogers, P.L., in *Better Living Through Innovative Biochemical Engineering*, Teo, W.K., Yap, M.G.S., and Oh, S.W.K., Eds., Continental Press, Singapore, 1994, p. 249.
37. Coughlin, R.W., Mahmoud, W.M., and El-Sayed, A.H., Enhanced bioconversion of toxic substances, U.S. Patent 663,828, 1992.
38. Rogers, P.L., Shin, H.S., and Wang, B., Biotransformation for ephedrine production, *Adv. Biochem. Eng. Biotechnol.*, 56, 33–59, 1997.
39. Voets, J.P., Vandamme, E.J., and Vlerick, C., Some aspects of the phenylacetylcarbinol biosynthesis by *Saccharomyces cerevisiae*, *Z. Allg. Mikrobiol.*, 13, 355–366, 1973.
40. Smith, P.F. and Hendlin, J., Mechanism of phenylacetylcarbinol synthesis by yeast, *J. Bacteriol.*, 65, 440–445, 1953.
41. Nikolova, P. and Ward, O.P., Production of L-phenylacetylcarbinol by biotransformation: product and by-product formation and activities of the key enzymes in wild-type and ADH isoenzyme mutants of *Saccharomyces cerevisiae*, *Biotechnol. Bioeng.*, 38, 493–498, 1991.
42. Long, A., James, P., and Ward, O.P., Aromatic aldehydes as substrates for yeast and yeast alcohol dehydrogenase, *Biotechnol. Bioeng.*, 33, 657–660, 1989.

43. Smith, P.F. and Hendlin, D., Further studies on phenylacetylcarbinol synthesis by yeast, *Appl. Microbiol.*, 2, 294–296, 1954.
44. Ose, S. and Hironaka, J., Studies on production of phenylacetylcarbinol by fermentation, *Proc. Int. Symp. Enzyme Chem.* (Tokyo), 2, 457–460, 1979.
45. Nikolova, P. and Ward, O.P., Effect of organic solvent on biotransformation of benzaldehyde to benzyl alcohol by free and silicone-alginate entrapped cells, *Biotech. Tech.*, 7, 897–902, 1993.
46. Mahmoud, W.M., El-Sayed, A.-H.M.M., and Caughlin, R.W., Production of L-phenylacetylcarbinol by immobilized yeast cells: I. Batch fermentation, *Biotechnol. Bioeng.*, 36, 47–54, 1990.
47. Mahmoud, W.M., El-Sayed, A.-H.M.M., and Caughlin, R.W., Production of L-phenylacetylcarbinol by immobilized yeast cells: II. Semi-continuous fermentation, *Biotechnol. Bioeng.*, 36, 55–60, 1990.
48. Nikolova, P. and Ward, O.P., Effect of support matrix on ratio of product to byproduct formation in L-phenylacetylcarbinol synthesis, *Biotechnol. Lett.*, 16, 7–10, 1994.
49. Shukla, V.B. and Kulkarni, P.R., Comparative studies on bioconversion of benzaldehyde to L-phenylacetylcarbinol (L-PAC) using calcium alginate- and barium alginate-immobilized cells of *Torulaspora delbrueckii*, *J. Chem. Technol. Biotechnol.*, 78, 949–951, 2003.
50. Barman, T.E., Pyruvate dehydrogenase, in *Enzyme Handbook*, vol. 2, Springer-Verlag, New York, 1969, p. 701.
51. Heider, J., Boll, M., Breese, K., Breinig, S., Ebenau-jehle, C., Feil, U., Gadon, N., Laempe, D., Leuthner, B., and Mohamed, M.E., Differential induction of enzymes involved in metabolism of aromatic compounds in the denitrifying bacterium *Thauera aromatica*, *Arch. Microbiol.*, 170, 120–131, 1998.
52. Nikolova, P. and Ward, O.P., Production of phenylacetylcarbinol by biotransformation using baker's yeast in two-phase systems, in *Biocatalysis in Non-Conventional Media*, Tramper, J., Ed., Elsevier, Amsterdam, 1992, pp. 675–680.
53. Nikolova, P. and Ward, O.P., Whole cell yeast biotransformations in two-phase systems: effect of solvent on product formation and cell structure, *J. Indust. Microbiol.*, 10, 169–177, 1992.
54. Laurence, G., Smallridge, A.J., and Trewhella, M.A., Lactate as an alternative to pyruvate in the yeast mediated preparation of PAC, *J. Mol. Catal., B Enzym.*, 19–20, 399–403, 2002.
55. Seely, R.J., Hageman, R.V., Yarus, M.J., and O'Sullivan, S.A., USP WO 90/04639; USP WO 90/04631, 1990.
56. Kostraby, M.M., Smallridge, A.J., and Trewhella, M.A., Yeast-mediated preparation of L-PAC in an organic solvent. *Biotechnol. Bioeng.*, 77, 827–831, 2002.
57. Seely, R.J., Heefner, D.L., Hageman, R.V., Yarus, M.J., and Sullivan, S.A., Process for making L-phenylacetylcarbinol (PAC), microorganisms for use in the process, and a method of preparing the microorganisms, U.S. Patent 5,312,742, 1994.
58. Nikolova, P. and Ward, O.P., Reductive biotransformation by wild type and mutant strains of *Saccharomyces cerevisiae* in aqueous–organic solvent systems, *Biotechnol. Bioeng.*, 39, 870–876, 1992.
59. Bruhn, H., Pohl, M., Grotzinger, J., and Kula, M.R., The replacement of Trp392 by alanine influences the decarboxylase/carboligase activity and stability of pyruvate decarboxylase from *Zymomonas mobilis*, *Eur. J. Biochem.*, 234, 650–655, 1995.
60. Engel, S., Vyazmensky, M., Vinogradov, M., Berkovich, D., Bar-Ilan, A., Qimron, U., Rosiansky, Y., Baral, Z., and Chipman, D., Role of conserved arginine in the mechanism of acetohydroxyacid synthase, *J. Biol. Chem.*, 279, 24803–24812, 2004.
61. Shin, H.S. and Rogers, P.L., Production of L-phenylacetylcarbinol (L-PAC) from benzaldehyde using partially purified pyruvate decarboxylase (PDC), *Biotechnol. Bioeng.*, 49, 52, 1996.
62. Iwan, P., Goetz, G., Schmitz, S., Hauer, B., Breuer, M., and Pohl, M., Studies on the continuous production of (R)-(–)-phenylacetylcarbinol in an enzyme-membrane bioreactor, *J. Mol. Catal. B Enzym.*, 11, 387–396, 2001.
63. Goetz, G., Iwan, P., Hauer, B., Breuer, M., and Pohl, M., Continuous production of (R)-phenylacetylcarbinol in an enzyme-membrane bioreactor using a potent mutant of pyruvate decarboxylase from *Zymomonas mobilis*, *Biotechnol. Bioeng.*, 74, 317–325, 2001.
64. Rosche, B., Breuer, M., Hauer, B., et al., Increased pyruvate efficiency in enzymatic production of (R)-phenylacetylcarbinol, *Biotechnol. Lett.*, 25, 847–851, 2003.

65. Leksawasdi, N., Chow, Y.Y.S., Breuer, M., Hauer, B., Rosche, B., and Rogers, P.L., Kinetic analysis and modeling of enzymatic (*R*)-phenylacetylcarbinol batch biotransformation process, *J. Biotechnol.*, 111, 179–189, 2004.
66. Rosche, B., Breuer, M., Hauer, B., and Rogers, P.L., Biphasic aqueous/organic biotransformation of acetaldehyde and benzaldehyde by *Zymomonas mobilis* pyruvate decarboxylase, *Biotechnol. Bioeng.*, 86, 788–794, 2004.
67. Barrowman, M.M. and Fewson, C.A., Phenylglyoxylate decarboxylase and pyruvate decarboxylase from *Acinetobacter calcoaceticus*, *Curr. Microbiol.*, 12, 235–240, 1985.
68. Barrowman, M.M., Harnett, W., Scott, A.J., Fewson, C.A., and Kusel, J.R., Immunological comparison of microbial TPP-dependent non-oxidative alpha-keto acid decarboxylases, *FEMS Microbiol. Lett.*, 34, 57–60, 1986.
69. Hegeman, G.D., Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*, *J. Bacteriol.*, 91, 1140–1159, 1966.
70. Hegeman, G.D., Benzoylformate decarboxylase (*Pseudomonas putida*), *Meth. Enzymol.*, 17A, 674–678, 1970.
71. Reynolds, L.J., Garcia, G.A., Kozarich, J.W., and Kenyon, G.L., Differential reactivity in the processing of [*para*-(halomethyl)benzoyl]formates by benzoylformate decarboxylase, a thiamin pyrophosphate dependent enzyme, *Biochemistry*, 27, 5530–5538, 1988.
72. Weiss, P.M., Garcia, G.A., Kenyon, G.L., and Clealand, W.N., Kinetics and mechanism of benzoylformate decarboxylase using C-13 and solvent deuterium isotope effects on benzoylformate and benzoylformate analogs, *Biochemistry*, 27, 2197–2205, 1988.
73. Wilcocks, R., Ward, O.P., Collins, S., Dewdney, N.J., Hong, Y., and Prosen, E., Acyloin formation by benzoylformate decarboxylase from *Pseudomonas putida*: a novel biotransformation, *Appl. Environ. Microbiol.*, 58, 1699–1704, 1992.
74. Wilcocks, R. and Ward, O.P., Factors affecting 2-hydroxypropiophenone formation by benzoylformate decarboxylases from *Pseudomonas putida*, *Biotechnol. Bioeng.*, 39, 1058–1063, 1992.
75. Ward, O.P., Wilcocks, R., Prosen, E., Collins, S., Dewdney, N.J., and Hong, Y., Acyloin formation mediated by benzoylformate decarboxylases, in *Microbial Reagents in Organic Synthesis*, Servi, S., Ed., Kluwer Publishers, Amsterdam, 1992, pp. 67–75.
76. Prosen, E., Ward, O.P., Collins, S., Dewdney, N.J., Hong, Y., and Wilcocks, R., Enantiospecific production of *S*-(–)-2-hydroxypropiophenone mediated by benzoylformate decarboxylase from *Acinetobacter calcoaceticus*, *Biocatalysis*, 8, 21–29, 1993.
77. Prosen, E. and Ward, O.P., Optimisation of reaction conditions for production of *S*-(–)-2-hydroxypropiophenone by *Acinetobacter calcoaceticus*, *J. Indust. Microbiol.*, 13, 287–291, 1994.
78. Demir, A.S., Dunwald, T., Iding, H., Pohl, M., and Muller, M., Asymmetric benzoin reaction catalyzed by benzoylformate decarboxylase, *Tetrahedron Asymmetry*, 10, 4769–4774, 1999.
79. Park, J.K. and Jung, J.Y., Production of benzaldehyde by encapsulated whole-cell benzoylformate decarboxylase, *Enzyme Microb. Technol.*, 30, 726–733, 2002.
80. Tsou, A.Y., Ransom, S.C., Geret, G.A., Buechter, B.D., Babbitt, P.C., and Kenyon, G.L., Mandelate pathway of *Pseudomonas putida* involving mandelate racemase, (*S*)-mandelate dehydrogenase and benzoylformate decarboxylase, and expression of benzoylformate decarboxylase in *Escherichia coli*, *Biochemistry*, 29, 9856–9867, 1990.
81. Hasson, M.S., Muscate, A., McLeish, M.J., Polovnikova, L.S., Gerlt, J.A., Kenyon, G.L., Petsko, G.A., and Ringe, D., The crystal structure of benzoylformate decarboxylase at 1.6Å resolution: diversity of catalytic residues in thiamine diphosphate-dependent enzymes, *Biochemistry*, 37, 9918–9930, 1998.
82. Lingen, B., Grotzinger, J., Kolter, D., Kula, M.-R., and Pohl, M., Improving the carbologase activity of benzoylformate decarboxylase from *Pseudomonas putida* by a combination of directed evolution and site-directed mutagenesis, *Prot. Eng.*, 15, 585–593, 2002.
83. Asakawa, T., Wada, H., and Yamano, T., Enzyme conversion of phenylpyruvate to phenylacetate, *Biochim. Biophys. Acta*, 170, 375–384, 1968.
84. Schneider, S., Mohamed, M.E., and Fuchs, G., Anaerobic metabolism of L-phenylalanine via benzoyl-CoA in the denitrifying bacterium *Thauera aromatica*, *Arch. Mikrobiol.*, 168, 310–320, 1998.

85. Vulalhan, Z., Morais, M.A., Tai, S.-L., Piper, M.D.W., and Pronk, J.T., Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol.*, 69, 4534, 2003.
86. Heider, J., Boll, M., Breese, K., Breinig, S., Ebenau-Jehle, C., Feil, U., Gadon, N., Laempe, D., Leuthner, B., and Mohamed, M.E., Differential induction of enzymes involved in metabolism of aromatic compounds in the denitrifying bacterium *Thauera aromatica*, *Arch. Microbiol.*, 170, 120–131, 1998.
87. Sentheshanmuganathan, S., The mechanism of the formation of higher alcohols from amino acids by *Saccharomyces cerevisiae*, *Biochem. J.*, 74, 568–576, 1960.
88. Guo, Z., Goswami, A., Mirfakhrae, D., and Patel, R.N., Asymmetric acyloin condensation catalysed by phenylpyruvate decarboxylase, *Tetrahedron Asymmetry*, 10, 4667–4675, 1999.
89. Schutz, A., Golbik, R., Tittmann, K., Svergun, D.I., Koch, M.H., Hubner, G., and Konig, S., Studies on structure–function relationships of indolepyruvate decarboxylase from *Enterobacter cloacae*, a key enzyme of the indole acetic acid pathway, *Eur. J. Biochem.*, 270, 2322–2331, 2003.
90. Schutz, A., Sandalova, T., Ricagno, S., Hubner, G., Konig, S., and Schneider, G., Crystal structure of thiamindiphosphate–dependent indolepyruvate decarboxylase from *Enterobacter cloacae*, an enzyme involved in the biosynthesis of the plant hormone indole-3-acetic acid, *Eur. J. Biochem.*, 270, 2312–2321, 2003.
91. Pang, S.S., Duggleby, R.G., Schowen, R.L., and Guddat, L.W., The crystal structure of *Klebsiella pneumoniae* acetolactate synthase with enzyme-bound cofactor and with an unusual intermediate, *J. Biol. Chem.*, 279, 2242–2253, 2004.
92. Zhang, G., Dai, J., Lu, Z., and Dunaway-Mariano, D., The phosphonopyruvate decarboxylase from *Bacteroides fragilis*, *J. Biol. Chem.*, 278, 41302–41308, 2003.
93. Graupner, M., Xu, H., and White, R.H., Identification of the gene encoding sulfopyruvate decarboxylase, an enzyme involved in biosynthesis of coenzyme, *M. J. Bacteriol.*, 182, 4862–4867, 2000.
94. Polnikova, E.S., McLeish, M.J., Sergienko, E.A., Burgner, J.T., Anderson, N.L., Bera, A.K., Jordan, G.L., and Hasson, M.S., Structural and kinetic analysis of catalysis of a thiamine diphosphate–dependent enzyme, benzoylformate decarboxylase, *Biochemistry*, 42, 1820–1830, 2003.
95. Duggleby, R.G., Pang, S.S., Yu, H., and Guddat, L.W., Systematic characterization of mutations in yeast acetohydroxyacid synthase: interpretation of herbicide resistance data, *Eur. J. Biochem.*, 270, 2895–2904, 2003.
96. Siegert, P., Pohl, M., Keen, M.M., Pogozheva, I.D., Kenyon, G.L., and McLeish, M.J., Exploring the substrate specificity of benzoylformate decarboxylase, pyruvate decarboxylase and benzaldehyde lyase, in *Thiamine: Catalytic Mechanisms in Normal and Disease States*, Jordan, F. and Patel, M.S., Eds., Marcel Dekker, New York, 2004, pp. 275–290.
97. Engel, S., Vyazmensky, M., Geresh, S., Barak, Z., and Chipman, D.M., Acetohydroxyacid synthase: a new enzyme for chiral synthesis of *R*-phenylacetylcarbinol, *Biotechnol. Bioeng.*, 83, 833–840, 2003.
98. Demir, A.S., Sesenoglu, O., Dunkelmann, P., and Muller, M., Benzaldehyde lyase-catalyzed enantioselective carbonylation of aromatic aldehydes with mono- and dimethoxy acetaldehyde, *Org. Lett.*, 5, 2047–2050, 2003.
99. Sanchez-Gonzalez, M. and Rosazza, J.P.N., Mixed aromatic acyloin condensations with recombinant benzaldehyde lyase: synthesis of alpha-hydroxydihydrochalcones and related alpha-hydroxyketones, *Synthetic. Catal.*, 345, 819–823, 2003.
100. Pohl, M., Sprenger, G.A., and Muller, M., A new perspective on thiamine catalysis, *Curr. Opin. Biotechnol.*, 15, 335, 2004.
101. Lingen, B., Kolter-Jung, D., Dunkelmann, P., Feldmann, R., Grotzinger, J., Pohl, M., and Muller, M., Alteration of the substrate specificity of benzoylformate decarboxylase from *Pseudomonas putida* by directed evolution, *Chem. Bio. Chem.*, 4, 721–726, 2003.

25 Synthesis of Chiral Alcohols with Carbonyl Reductase Library and Robust NAD(P)H Regenerating System

Hiroaki Yamamoto and Akinobu Matsuyama

CONTENTS

25.1	Introduction	623
25.2	(<i>R</i>)-1,3-Butanediol and (<i>S</i>)-Specific Alcohol Dehydrogenase	624
25.3	Synthesis of Several Chiral Alcohols with CpSADH	627
25.4	Innovative Screening of Ethyl 4-Chloroacetoacetate Reductases and Synthesis of Ethyl (<i>S</i>)-4-Chloro-3-Hydroxybutanoate	628
25.5	Synthesis of Several Chiral Alcohols with PfODH	632
25.6	α -Keto Acid Reductases for Synthesis of Chiral α -Hydroxy Acids	633
25.7	Synthesis of (<i>R</i>)- α -Hydroxy Acids with α -Keto Acid Reductases	634
25.8	Robust NADH Regenerator, McFDH-26	637
25.9	Remarks	640
	References	640

25.1 INTRODUCTION

Optically pure compounds are useful as building blocks and intermediates of pharmaceuticals and fragrances. Chiral compounds can be synthesized mainly by the following three methods:

1. A chiral pool method to obtain them by chemical conversion of naturally occurring chiral compounds such as L-amino acids, D-sugars, and organic acids [1]
2. An optical resolution of racemic mixture to obtain them by preferential crystallization [2], diastereomeric resolution [3], kinetic resolution [4], and chromatographic separation with chiral stationary phases [5]
3. An asymmetric synthesis method with chiral auxiliaries [6], metal-catalysts [7], and biocatalysts [8]

The optimal method to obtain chiral compounds is selected according to the quantity needed, allowable lead time, and cost. The biocatalytic process has several advantages: (1) it is generally highly regio- and stereoselective; (2) it is performed at a moderate temperature in aqueous solution; (3) biocatalysts can be reproduced easily and inexpensively; and (4) it is low environmental burden.

We have been investigating the synthesis of chiral alcohols by asymmetric reduction with biocatalysts because its theoretical yield is 100% without any particular recycle process. There have been, however, several problems in the manufacture of chiral alcohols by biological-asymmetric reduction: (1) low enzyme activity; (2) sometimes low optical purity and its variance; (3) cost of coenzyme and its regeneration; (4) low concentration of product accumulated; (5) time-consuming for development, from enzyme screening to process development; and so on. As a strategy to overcome these problems, we have constructed the cloned enzyme library consisting of recombinant whole-cell biocatalysts. Low enzyme activity and inadequate optical purity have been overcome by high expression of excellent enzymes that have strict substrate- and enantioselectivity as well as toughness, in a heterologous host, especially *Escherichia coli*. *E. coli* is a suitable host for asymmetric reduction because it rarely produces carbonyl reductases, which lead to decrease in optical purity. Subjects about coenzymes have been significantly, although not perfectly, overcome using whole recombinant biocatalysts coexpressing glucose dehydrogenase (GDH) [9] and formate dehydrogenase (FDH) [10] as NAD(P)H-regenerating systems with a carbonyl reductase. Whole-cell biocatalysis enabled an efficient use of intracellular coenzymes without addition of extra cofactor [11]. A cloned enzyme library could shorten development time. If an appropriate enzyme were obtained by cloned enzyme library screening, we could advance to process development without improvements of microorganism and culture conditions, enzyme purification, gene cloning, optimization of gene expression, and so on. In this chapter, the procedure of constructing a cloned enzyme library and its application are described.

25.2 (R)-1,3-BUTANEDIOL AND (S)-SPECIFIC ALCOHOL DEHYDROGENASE

(R)-1,3-butanediol (BDO) is used as a raw material for the production of azetidinone derivatives [12,13], which are intermediates for the syntheses of penem and carbapenem antibiotics [14]. It may be produced by kinetic resolution, asymmetric reduction of 4-hydroxy-2-butanone [15], and enantioselective oxidation of racemic 1,3-BDO [16]. (R)-1,3-BDO has been manufactured by enantioselective oxidation on an industrial scale, as 4-hydroxy-2-butanone is more expensive and more toxic to microorganisms than commercial racemic 1,3-BDO for cosmetic use.

The enzyme responsible for the enantioselective oxidation of (S)-1,3-BDO was purified from *Candida parapsilosis* and characterized [17]. The enzyme was NAD⁺-dependent (S)-specific alcohol dehydrogenase (ADH) and designated as CpSADH. It catalyzed the enantioselective oxidation of several (S)-alcohols such as (S)-2-butanol, (S)-2-octanol, and (S)-1-phenylethanol, and (S)- β -hydroxy esters, such as ethyl (S)-3-hydroxybutanoate, and the asymmetric reduction of several ketones and β -oxo esters. A gene encoding CpSADH was cloned and highly expressed in *E. coli* [18]. The gene consists of 1009 nucleotides encoding a protein with a molecular weight of 35,964. CpSADH is assumed to belong to group I ADH, long-chain, zinc-dependent ADH [19]. The properties of CpSADH and comparison with several (S)-specific ADHs are summarized in Table 25.1 and its substrate specificity is shown in Table 25.2.

The enzymatic synthesis of (R)-1,3-BDO was developed with whole recombinant *E. coli* cells expressing CpSADH as shown in Figure 25.1 [27]. There are only a few reports on efficient production systems of optically active alcohols by enantioselective oxidation using recombinant *E. coli* cells expressing an ADH. The optimal pH for the reaction was 6.8, which suggested that NAD⁺ regeneration is a rate-limiting step. The optimal pH and the stable pH for the regeneration of the coenzyme NAD⁺ were around 6.8 because the optimal pH of CpSADH for oxidation of (S)-1,3-BDO and its stable pH were around 9.0.

TABLE 25.1
Properties of (S)-Specific Secondary Alcohol Dehydrogenases (ADHs)

Name	CpSADH	CpCR	CPAR	NfsADH	ReCR	RrSADH
Origin	<i>Candida parapsilosis</i> IFO 1396	<i>C. parapsilosis</i> DSM 70125	<i>Corynebacterium</i> sp. ST-10	<i>Nocardia fusca</i> AKU 2123	<i>Rhodococcus erythropolis</i> DSM 743	<i>R. ruber</i> DSM 44551
<i>Mr</i>						
Native	140,000	136,000	155,000	150,000	161,000	62,000
Subunit	40,000	67,000	42,000	39,000	40,000	38,000
ORF	336 aa	Not cloned	385 aa	Not cloned	348 aa	Not cloned
Optimum for oxidation						
Temperature (°C)	50	52–56	ND	60	ND	54
pH	9.0	7.8–8.6	10.5	8.5–9.5	9.5	9.0
Optimum for reduction						
Temperature (°C)	ND	36–40	ND	65	40	54
pH	6.0	6.5–7.2	7.0	5.5–6.5	5.5	6.5–7.5
Cofactor	NAD ⁺ /NADH	NAD ⁺ /NADH	NAD ⁺ /NADH	NAD ⁺ /NADH	NAD ⁺ /NADH	NAD ⁺ /NADH
Activity						
Substrate	(S)-1,3-Butanediol	Ethyl 5-oxohexanoate	Phenylacetaldehyde	2-Hexanol	Ethyl 3-oxobutanoate	1-Phenylethanol
Specific activity (U/mg)	244	1,855	64	92	269	17.5
Superfamily	SDR	Unknown	MDR	Unknown	MDR	Unknown
References	[17,18]	[20]	[21,22]	[23]	[24,25]	[26]

Mr, relative molecular mass; ND, not determined; SDR, short-chain ADH; MDR, medium-chain ADH.

TABLE 25.2
Substrate Specificity of CpSADH

Substrate	Concentration (mM)	Cofactor	pH	Relative Activity (%)
<i>Oxidation</i>				
(<i>S</i>)-1,3-Butanediol	50	NAD ⁺	9.0	100
	50	NADP ⁺	9.0	3.4
(<i>R</i>)-1,3-Butanediol	50	NAD ⁺	9.0	1.7
Methanol	100	NAD ⁺	9.0	1.0
Ethanol	100	NAD ⁺	9.0	5.4
1-Propanol	100	NAD ⁺	9.0	8.5
2-Propanol	100	NAD ⁺	9.0	337
2-Butanol	100	NAD ⁺	9.0	244
(<i>S</i>)-2-Butanol	50	NAD ⁺	9.0	562
(<i>R</i>)-2-Butanol	50	NAD ⁺	9.0	18.8
2-Pentanol	100	NAD ⁺	9.0	191
3-Pentanol	100	NAD ⁺	9.0	58.3
2-Hexanol	50	NAD ⁺	9.0	156
2-Octanol	5	NAD ⁺	9.0	220
(<i>S</i>)-2-Octanol	5	NAD ⁺	9.0	381
(<i>R</i>)-2-Octanol	5	NAD ⁺	9.0	0
Cyclohexanol	20	NAD ⁺	9.0	297
(<i>S</i>)-1-Phenylethanol	50	NAD ⁺	9.0	502
(<i>R</i>)-1-Phenylethanol	50	NAD ⁺	9.0	6.4
Methyl (<i>S</i>)-3-hydroxybutanoate	50	NAD ⁺	9.0	266.9
Methyl (<i>R</i>)-3-hydroxybutanoate	50	NAD ⁺	9.0	9.9
Ethyl (<i>S</i>)-3-hydroxybutanoate	20	NAD ⁺	9.0	736.5
Ethyl (<i>R</i>)-3-hydroxybutanoate	20	NAD ⁺	9.0	3.2
<i>Reduction</i>				
4-Hydroxy-2-butanone	100	NADH	6.0	100
Acetone	100	NADH	6.0	299
2-Butanone	100	NADH	6.0	243
Ethyl acetoacetate	100	NADH	6.0	465
Ethyl 4-chloroacetoacetate	100	NADH	6.0	341
Acetophenone	20	NADH	6.0	296
Phenacyl chloride	10	NADH	6.5	18.8
3-Quinuclidinone	20	NADH	6.0	13.0

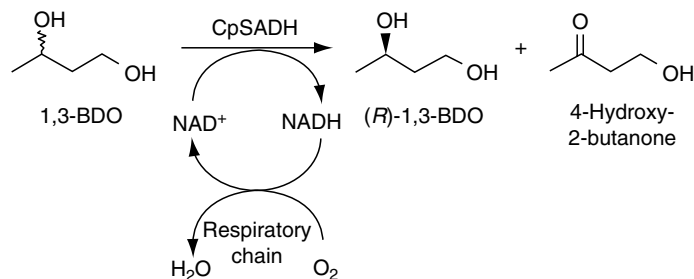


FIGURE 25.1 Enzymatic synthesis of (*R*)-1,3-butanediol using recombinant whole cells of *Escherichia coli* expressing CpSADH. 1,3-BDO, 1,3-butanediol.

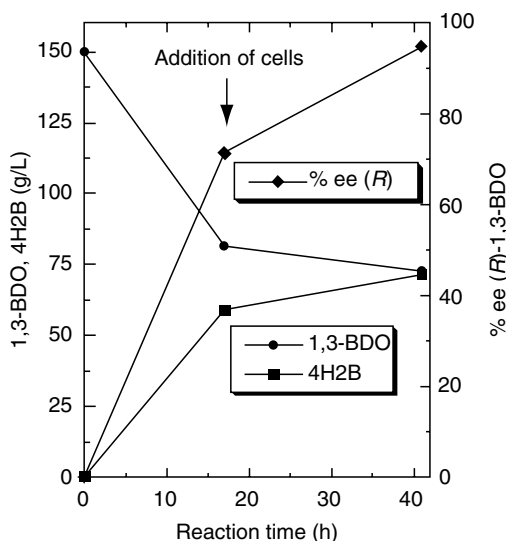


FIGURE 25.2 Synthesis of (*R*)-1,3-butanediol with whole *E. coli* cells expressing CpSADH. The reaction mixture (25 mL) containing 15% 1,3-butanediol and cells obtained from 25 mL culture in YT medium (bacto-tryptone 10 g, bacto-yeast extract 5 g, and NaCl 5 g) and 100 mM potassium phosphate (pH 6.8) at 30°C with shaking; after 17 h incubation cells obtained from 25 mL culture were added and further incubated. 1,3-BDO, 1,3-butanediol; 4H2B, 4-hydroxy-2-butanone.

NAD⁺ was thought to be regenerated by aerobic respiratory chain in *E. coli*. *E. coli* has no cytochrome *c* and no equivalent to the mitochondrial Complex III (*bc1* complex) or Complex IV (cytochrome *c* oxidase). Instead, two terminal oxidases in the *E. coli* cytoplasmic membrane, cytochrome *o* and cytochrome *d* complexes, oxidize ubiquinol and directly reduce molecular oxygen to water, concomitantly generating an electrochemical proton gradient across the membrane [28]. The optimal pH of NADH oxidase of *E. coli* membrane vesicles was reported to be 7.5 to 8.0 [29]. From these reports, the optimal pH for the synthesis of (*R*)-1,3-BDO using whole recombinant *E. coli* cells seems to result from the stability of NAD⁺ and/or the NADH oxidase complex. Furthermore, in a reaction containing more than 7% 1,3-BDO, the cessation of enantioselective oxidation was observed. The cessation of the reaction resulted not from the inactivation of CpSADH but from the loss of NAD⁺ and/or NAD⁺-regeneration activity. The cessation was overcome by the addition of a fresh YT medium or its component, a yeast extract, to the reaction mixture. The addition of a YT-medium afforded cell growth during the reaction and is thought to supply fresh biocatalysts. Moreover, the yield of (*R*)-1,3-BDO reached 72.6 g/L, in 48.4% reaction yield (maximum theoretical yield is 50%) and 95% ee at 15% racemate by the addition of fresh cells after the 17 h incubation, as shown in Figure 25.2.

25.3 SYNTHESIS OF SEVERAL CHIRAL ALCOHOLS WITH CpSADH

Several optically active alcohols were synthesized with *E. coli* cells expressing CpSADH. Two NADH-regeneration systems were used for the asymmetric reduction in *E. coli*, as shown in Figure 25.3. One is NAD⁺ reduction coupled with the oxidation of 2-propanol by CpSADH itself, and the other is that coupled with the oxidation of formate by formate dehydrogenase (McFDH) from *Mycobacterium vaccae* [30,31]. In both systems only intracellular NAD⁺ was used.

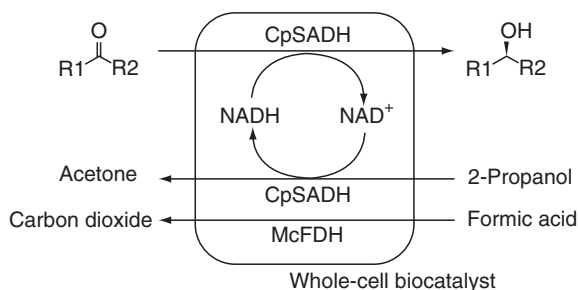


FIGURE 25.3 Whole-cell biocatalyst system containing CpSADH and NADH regenerator.

Ethyl (*R*)-4-chloro-3-hydroxybutanoate [(*R*)-ECHB] is a chiral compound that is useful for the synthesis of biologically and pharmacologically important materials: (*R*)-carnitine [32,33], (*R*)-4-amino-3-hydroxybutyric acid [34], and (*R*)-4-hydroxy-2-pyrrolidone [35]. (*R*)-ECHB was synthesized from ethyl 4-chloroacetoacetate (ECAA) and 2-propanol with whole recombinant *E. coli* cells expressing CpSADH only [11]. The ratio of 2-propanol as a cosubstrate to ECAA as a substrate was optimized in the reaction mixture. Two molar excesses of 2-propanol over ECAA gave the best result, and 1.2-fold excess also gave similar results. Surprisingly, CpSADH had little dehydrogenase activities for (*R*)-ECHB and (*S*)-ECHB, although it showed a high activity for ethyl (*S*)-3-hydroxybutanoate without chlorine substitution at the 4-position. The extremely efficient regeneration of NADH was due to the unique properties of CpSADH, which functioned as an exclusive reductase for ECAA. The yield of (*R*)-ECHB reached 36.6 g/L in 95.2% conversion yield with a >99% ee under optimized reaction conditions comprising 3.8% ECAA, 2 molar excesses of 2-propanol over ECAA, 200 mM potassium phosphate (pH 6.5), and recombinant *E. coli* cells expressing CpSADH, at 20°C.

Several optically active alcohols, furthermore, were synthesized with whole *E. coli* cells expressing CpSADH only or CpSADH and McFDH as an NADH regenerator, and some examples are shown in Figure 25.4. CpSADH produced (*S*)-1,3-BDO from 4-hydroxy-2-butanone efficiently by asymmetric reduction, and (*R*)-1,3-BDO from the racemate by enantioselective oxidation. Optical purity of alcohols obtained was more than 99% ee. 2-Acetylbutyrolactone, which had a chiral center at α -position, was reduced to (1'*S*,2*R*)-2-(1-hydroxyethyl)butyrolactone (HEBL) in 99% ee and 95% de by *syn*-reduction accompanying dynamic resolution. These results show that CpSADH is a powerful and versatile tool to produce optically active (*S*)-alcohols. CpSADH, also, enantioselectively catalyzed asymmetric reduction of α -haloketones such as phenacyl chloride and *m*-chlorophenacyl chloride. Although the reactions were catalyzed at low concentrations of α -haloketones in a biphasic system, they could not accumulate (*R*)- α -haloalcohols at an industrial level. α -Haloketones were found to be inhibitory and suicide substrates to CpSADH. Further investigation toward directed evolution and protein engineering is needed to manufacture (*R*)- α -haloalcohols with CpSADH.

25.4 INNOVATIVE SCREENING OF ETHYL 4-CHLOROACETOACETATE REDUCTASES AND SYNTHESIS OF ETHYL (*S*)-4-CHLORO-3-HYDROXYBUTANOATE

Ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-ECHB] is a chiral synthon that is useful for the synthesis of pharmacologically active compounds, such as hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors [36] and 4-hydroxypyrrolidone [37]. The asymmetric

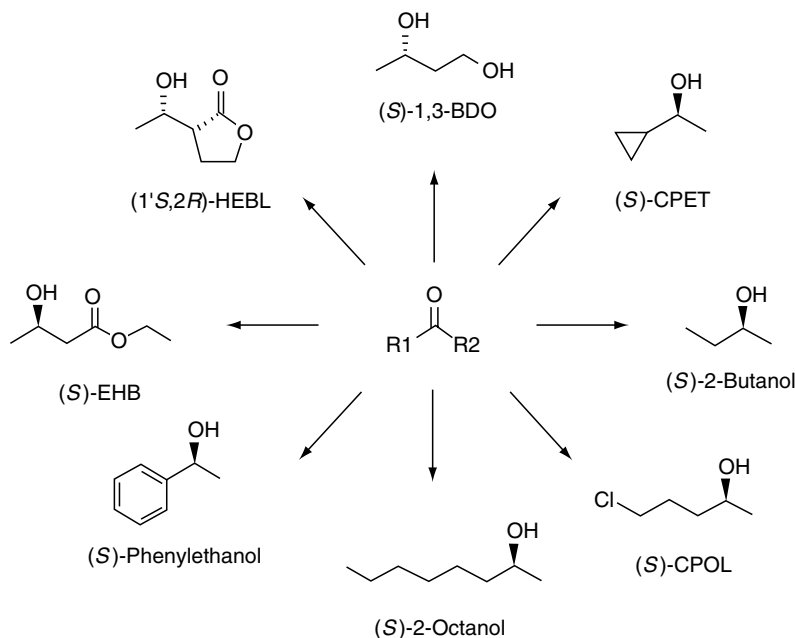


FIGURE 25.4 Synthesis of optically active alcohols with CpSADH. (S) -1,3-BDO, (S) -1,3-butenediol; (S) -CPET, (S) -cyclopropylethanol; (S) -CPOL, (S) -5-chloro-2-pentanol; (S) -EHB, ethyl (S) -3-hydroxybutanoate; $(1'S,2R)$ -HEBL, $(1'S,2R)$ -2-(1'-hydroxyethyl)butyrolactone.

reduction using enantioselective oxidoreductases is a practical method for the production of (S) -ECHB. Several enzymes reducing ECAA to (S) -ECHB have been found and purified from *Saccharomyces cerevisiae* [38], *Geotrichum candidum* [39], *Sporobolomyces salmonicolor* [40], *C. macedoniensis* [41], and *C. magnoliae* [42].

We found several classes of ECAA reductases by innovative screening methods. First, we screened microorganisms that reduced ECAA asymmetrically to synthesize (S) -ECHB by a conventional method and selected *Kluyveromyces lactis* NRIC 1329 as the best producer of (S) -ECHB. This strain, however, was unsuitable for the production of (S) -ECHB on an industrial scale from the viewpoints of optical purity of ECHB produced and productivity. We found that the enzyme which participated in the asymmetric reduction of ECAA in *K. lactis* was a type-IB fatty acid synthase (FAS-IB) [43]. Type FAS-IB was not appropriate for the overexpression in heterologous hosts, such as *E. coli*, as it is a complex protein comprising $\alpha_6\beta_6$ subunits and has a multifunctional enzyme that catalyzes eight kinds of reactions for fatty acids synthesis [44].

Second, we screened ECAA reductases from functional homology. We noticed that there were several types of FAS (IA, IB, IC, and II) among living organisms, as shown in Table 25.3 [45]. Type II FAS, in particular, consisted of several small monofunctional enzymes, and some bacteria, viruses, and higher plants were reported to have type II FAS components. The reduction of ECAA by type IB FAS seemed to be analogous to that of β -ketoacyl-ACP by general FAS. In type II FAS, the reduction of β -ketoacyl-ACP was catalyzed by β -ketoacyl-ACP reductase (KR, E.C.1.1.1.100) [46] encoded by *fabG* [47]. We examined the capabilities of β -ketoacyl-ACP reductases for synthesis of (S) -ECHB from ECAA.

Third, we screened ECAA reductases from amino acid sequence homology. A similarity search for the amino acid sequence of KR from *E. coli* with public databases showed significant similarity to acetoacetyl-CoA reductases (ARs, E.C.1.1.1.36) encoded by *phbB*,

TABLE 25.3
Comparison of Properties of Fatty Acid Synthase

Type	IA	IB	IC	II
E.C. No.	2.3.1.85	2.3.1.86	2.3.1.86	—
Structure	α_2	$\alpha_6\beta_6$	α_6	—
Molecular weight	500K	2400K	1390K	—
Subunit size	α : 250K —	α : 213K β : 203K	α : 250K —	— —
Function	Localization			Gene
Acyl-carrier protein (ACP)	α	α	α	<i>acpP</i>
ACP S-acetyltransferase (AT)	α	β	α	—
ACP S-malonyltransferase (MT)	α	β	α	<i>fabD</i>
β -Ketoacyl-ACP synthase (KS)	α	α	α	<i>fabB,C</i> <i>fabF,J</i> <i>fabH</i>
β -Ketoacyl-ACP reductase (KR)	α	α	α	<i>fabG</i>
β -Hydroxyacyl-ACP dehydratase (DH)	α	β	α	<i>fabA</i>
Enoyl-ACP reductase (ER)	α	β (FMN)	α (FMN)	<i>fabI</i>
Acyl-ACP hydrolase (TE)	α	—	—	<i>tes</i>
Palmitoyl transferase (PT)	—	β	α	—

which play a physiological role for poly- β -hydroxybutyric acid (PHB) [48]. We also examined the capabilities of AR to synthesize (S)-ECHB. Two KR-genes from *E. coli* (EcKR1) [47,49] and *Bacillus subtilis* (BsKR1) [50], and two AR-genes from *Ralstonia eutropha* (ReAR1) [51,52] and *Zoogloea ramigera* (ZrAR1) [53,54] were cloned and expressed in *E. coli* and their substrate specificities and enantioselectivities were examined. All four enzymes catalyzed the asymmetric reduction of ECAA to synthesize (S)-ECHB as expected, but the optical purity of ECHB obtained varied as shown in Table 25.4 [55]. ReAR1 was selected as the best enzyme and a plasmid coexpressing ReAR1, and a GDH from *B. subtilis* (BsGDH) [56],

TABLE 25.4
Substrate Specificities and Enantioselectivities of β -Ketoacyl-ACP Reductases and Acetoacetyl-CoA Reductases

Enzyme	Relative Activity (%)								
	ECAA		AcAc-SCoA		EAA		(R)-ECHB	(S)-ECHB	(S)-ECHB
	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADP ⁺		ee (%)
EcKR1	100	<1	116	ND	1.9	ND	<1	<1	93.7
BsKR1	100	<1	22.3	ND	4.8	ND	1.3	1.3	98.0
ReAR1	100	2.3	39.3	ND	1.3	ND	<1	<1	99.3
ZrAR1	100	<1	284	ND	38.4	ND	<1	<1	99.5

The relative activities for substrates were expressed as percentage to the activity for ECAA. AcAc-SCoA, acetoacetyl-CoA; EAA, ethyl acetoacetate; ND, not determined.

pSG-AER1, was constructed. (*S*)-ECHB was produced using whole *E. coli* cells harboring pSG-AER1 on a 1.2 L jar scale. The synthesis of (*S*)-ECHB was performed at 25°C and pH 6.5 in a working volume of 750 mL, as the substrate, ECAA, was unstable at more than pH 7.0 in an aqueous solution and at a higher temperature. To keep the concentration of ECAA in the reaction mixture at a low level, the initial concentration of ECAA was 2% (w/v), and ECAA was fed into the reaction mixture for 4 h at the rate of 7.5 g/L/h after 2 h of initiation. After 18 h, the amount of ECHB reached 48.7 g/L with 99.8% ee (*S*).

Furthermore, we screened and discovered two kinds of NADH-dependent ECAA reductases that had not been found. NADH-dependent ADH/carbonyl reductases have several advantages for manufacturing chiral compounds: (1) NAD(H) was chemically more stable than NADP(H); (2) the intracellular concentrations of NAD(H) were reported to be higher than those of NADPH in several microorganisms [57,58]; (3) FDH can be utilized for NADH regeneration instead of GDH. One was an NADH-dependent carbonyl reductase from *K. aestuarii* (KaCR1). KaCR1 was purified to homogeneity and its apparent subunit molecular mass was 32,000 on an SDS-PAGE [59]. The enzyme mainly used NADH as an electron donor, and NADHPH could replace NADH with only 6.4% of activity. The enzyme had maximal activity at pH 5.0 to 5.5 and 45°C for the reduction of ECAA but no oxidative activity of (*S*)- or (*R*)-ECHB. These findings suggested that the enzyme functioned as an exclusive reductase. KaCR1 had restricted substrate specificity: it showed high activities for 4-chloroacetoacetate esters such as ethyl ester and methyl ester, and α,β -diketones such as 2,3-butanedione and 2,3-pentanedione; however, it showed none or little activity for ethyl acetoacetate without chlorine substitution at the 4-position, simple ketones, or aldehydes such as pyridine-3-aldehyde, a typical substrate for the aldo-keto reductase superfamily enzymes. The KaCR1 gene was cloned and was shown to contain an open reading frame of 876 nucleotides encoding a polypeptide of 292 amino acid residues with a calculated molecular weight of 31,687. The other was a novel (*R*)-specific ADH, (*R*)-2-octanol dehydrogenase (PfODH), from a methanol-assimilating yeast, *Pichia finlandica*. It was difficult to discover PfODH by conventional screening because it was a minor enzyme similar to ECAA reductases in *P. finlandica*. We screened using native polyacrylamide gel electrophoresis and differential activity staining. We found PfODH as an enzyme that had activities both for the reduction of ECAA and for the oxidation of (*R*)-2-octanol, and no activity for the oxidation of (*S*)-2-octanol in gels. PfODH was purified to homogeneity and characterized, and its apparent subunit molecular mass was 30,000 on an SDS-PAGE [60]. The enzyme was reversible and had maximal activity at pH 10.5 and 50°C for the oxidation of (*R*)-2-octanol, and at pH 6.0 and 55 to 60°C for the reduction of ECAA. The enzyme had very broad substrate specificity. The PfODH gene was cloned and was shown to contain an open reading frame of 765 nucleotides encoding a polypeptide of 254 amino acid residues with a calculated molecular weight of 27,143.

We obtained several kinds of ECAA reductases by innovative screening methods such as functional homology search, structural homology search, and differential activity staining. Properties of several ECAA reductases containing KaCR1 and PfODH are summarized in [Table 25.5](#).

Both KaCR1 and PfODH reduced ECAA asymmetrically to synthesize (*S*)-ECHB in >99% ee. Coexpression plasmids of KaCR1 and PfODH with McFDH-26, pSFR426, and pSF-PFO3, respectively, were constructed for the use of whole-cell biocatalysts. McFHD-26 is a mutant FDH from *M. vaccae* having three amino acid substitutions [61], and the construction McFDH-26 is described in detail in [Section 25.8](#). The synthesis of (*S*)-ECHB was examined with *E. coli* harboring pSFR426 and pSF-PFO3. Reaction conditions at a mini-jar scale were the same as those for *E. coli* cells harboring pSG-AER1. Using *E. coli* cells harboring pSFR426 and pSF-PFO3, the amount of ECHB reached 49.9 g/L in >99%

TABLE 25.5
Comparison of Properties of (S)-ECHB-Forming ECAA Reductases

Name	PfODH	KaCR1	ReAR1	KICR1 (FAS α)	CmCR-S1	LbRADH
Origin	<i>Pichia finlandica</i>	<i>Kluyveromyces aestuarii</i>	<i>Ralstonia eutropha</i>	<i>K. lactis</i>	<i>Candida magnoliae</i>	<i>Lactobacillus brevis</i>
E.C.	1.1.1.1	—	1.1.1.36	2.3.1.86	—	1.1.1.2
Cofactor	NADH	NADH	NADPH	NADPH	NADPH	NADPH
Mr						
Native	83,000	85,000	84,000	>800,000	76,000	104,000
Subunit	30,000	32,000	23,000	190,000	32,000	27,000
ORF	254 aa (27,100)	292 aa (31,700)	246 aa (26,400)	—	283 aa (30,400)	252 aa (26,800)
Optimum for reduction						
Temperature	55–60	45	—	45	50–55	50
pH	6.0	5.0–5.5	—	6.5	5.5–6.5	7.0
Activity						
Substrate	(R)-2-Octanol	ECAA	Acetoacetyl-CoA	ECAA	ECAA	Acetophenone
Specific activity (U/mg)	91.1	28.6	44.0	50.8	13.5	489
Substrate specificity	Broad	Narrow	Narrow	Narrow	Broad	Broad
Reversibility	Reversible	Irreversible	Irreversible	Irreversible	Irreversible	Reversible
Superfamily	SDR	SDR	SDR	SDR	SDR	SDR
References	[60]	[59]	[51]	[43]	[42]	[24]

Mr, relative molecular mass; ND, not determined; SDR, short-chain ADH; MDR, medium-chain ADH.

ee (S) and 49.4 g/L in >99% ee, respectively. The discovery of NADH-dependent ECAA reductases, such as KaCR1 and PfODH, and the use of McFDH-26 potentially led to the manufacturing of (S)-ECHB with low environmental burden.

25.5 SYNTHESIS OF SEVERAL CHIRAL ALCOHOLS WITH PfODH

Application of KaCR1 was limited because of its narrow substrate specificity compared with that of PfODH, which has very broad substrate specificity and high stability. Several chiral alcohols such as (R)-alcohol, (S)- α -haloalcohol, and (R)-3-hydroxy esters, were synthesized in >99% ee from corresponding ketones with recombinant *E. coli* cells harboring pSF-PFO3 as shown in Figure 25.5. 2,3-Difluoro-6-nitro-[[*(R)*-2-hydroxypropyl]oxy]benzene, (R)-FNHB, was a key intermediate for the synthesis of an antibacterial agent, Levofloxacin. (R)-FNHB was efficiently synthesized with 86% yield and >99% ee from the corresponding ketone at 100 g/L input. 2-Acetylbutyrolactone, which had a chiral center at α -position of the carbonyl group, was reduced to (1'*R*,2*S*)-2-(1-HEBL) in 99% ee and 90% de by *syn*-reduction accompanying dynamic resolution. As the PfODH had a high tolerance to α -haloketones, it was used to synthesize several α -haloalcohols such as (S)-2-chloro-1-phenylethanol, (S)-2-chloro-1-(4'-fluorophenyl)ethanol, and (S)-2-chloromethyl-3-pyridinemethanol in >99% ee.

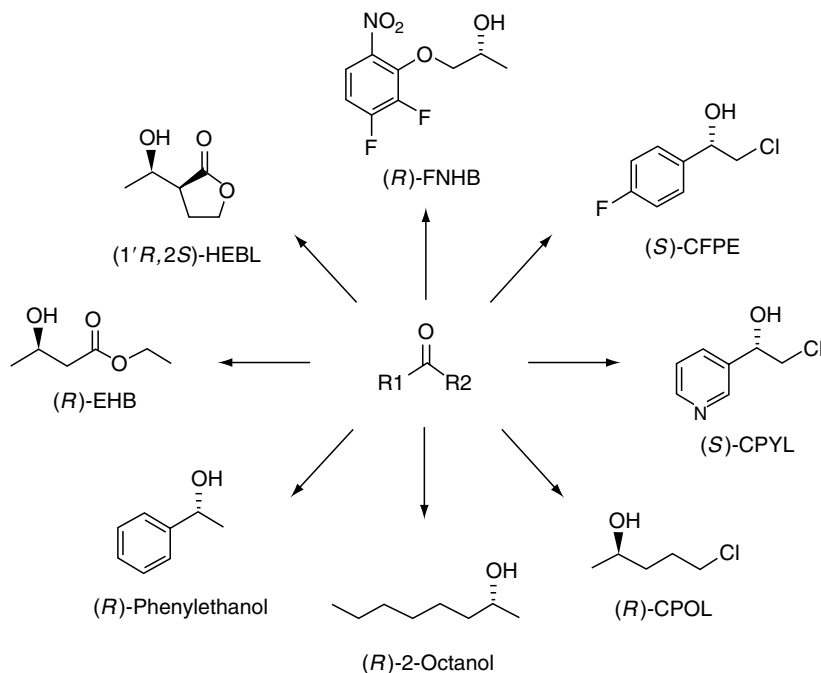


FIGURE 25.5 Synthesis of optically active alcohols with PfODH. (*R*)-FNHB, 2,3-difluoro-6-nitro-[[(*R*)-2-hydroxypropyl]oxy]benzene; (*S*)-CFPE, (*S*)-2-chloro-1-(4'-fluorophenyl)ethanol; (*S*)-CPYL, (*S*)-2-chloromethyl-3-pyridinemethanol; (*R*)-CPOL, (*R*)-5-chloro-2-pentanol; (*R*)-EHB, ethyl (*R*)-3-hydroxybutanoate; (1'*R*,2*S*)-HEBL, (1'*R*,2*S*)-2-(1'-hydroxyethyl)butyrolactone.

These compounds were easily transformed to corresponding chiral epoxides for versatile chemical synthesis. These results show that PfODH is a powerful enzyme for synthesis of optically active alcohols, especially α -haloalcohols.

25.6 α -KETO ACID REDUCTASES FOR SYNTHESIS OF CHIRAL α -HYDROXY ACIDS

Chiral α -hydroxy acids are useful as building blocks and intermediates for the synthesis of several pharmaceuticals. (*R*)-benzyl lactate ethyl ester is useful as an intermediate for the synthesis of angiotensin-converting enzyme (ACE) inhibitor [62,63]. (*R*)-Phenyllactic acid is useful as a precursor of anthelmintic cyclodepsipeptide PF1022A produced by *Mycelia sterilia* [64]. (*R*)-2-Chloromandelic acid, designated as (*R*)-2-CMA, is useful as an intermediate for the synthesis of an inhibitor of platelet aggregation and a fungicide [65,66]. (*R*)-2-CMA could be synthesized by diastereomeric resolution with chiral amines [67–69], enzymatic resolution [70], and asymmetrically enzymatic synthesis with hydroxynitrile lyases [71], D-lactate dehydrogenase [72,73], and D-hydroxyisocaproate dehydrogenases [74].

We found unique α -keto acid reductases that had wide substrate specificity by screening with a variety of substrates such as benzylpyruvic acid and 2-chlorobenzoylformic acid. At first, we screened microorganisms that catalyzed asymmetric reduction of benzylpyruvic acid to synthesize (*R*)-benzyl lactic acid. *Leuconostoc oenos* (*L. dextranicum* subsp. *vinerium*) was selected as the best microorganism, and an enzyme responsible for the asymmetric reduction was purified to homogeneity [75]. The enzyme, which was designated as LoKAR1, catalyzed

NADPH-dependent reduction of benzylpyruvic acid to synthesize (*R*)-benzylactic acid, irreversibly. Its apparent molecular mass was 47,000 on gel filtration and 33,000 on SDS-PAGE. The enzyme had maximal activity at pH 6.5 to 7.0 and 40°C for the reduction of benzylpyruvic acid. LoKAR1 had higher activity for substrates having a long-chain substituent, such as 2-oxo-4-phenylbutyric acid and 2-oxo-5-phenylpentanoic acid, than phenylpyruvic acid and benzoylformic acid. The LoKAR1 gene was cloned and was shown to contain an open reading frame of 924 nucleotides encoding a polypeptide of 307 amino acid residues with a calculated molecular mass of 33,049 [76].

Microorganisms were also screened that catalyzed asymmetric reduction of 2-chlorobenzoylformic acid to synthesize (*R*)-2-chloromandelic acid. *L. mesenteroides* subsp. *dextraniticum* was selected as the best microorganism, and an enzyme responsible for the asymmetric reduction was purified to homogeneity [77]. The enzyme, which was designated as LmKAR1, catalyzed NADH-dependent reduction of 2-chlorobenzoylformic acid to synthesize (*R*)-2-chloromandelic acid, irreversibly. Its apparent molecular mass was 62,600 on gel filtration and 34,100 on SDS-PAGE. The enzyme had maximal activity at pH 5.5 to 6.0 and 50°C for the reduction of 2-chlorobenzoylformic acid. LmKAR1 had quite contrasting substrate specificity to LoKAR1. It had extremely high activity for substrates having a bulky and/or branched substituent at a proximal position of the carbonyl group, e.g., 2,620 U/mg for 2-chlorobenzoylformic acid. LmKAR1 gene was cloned and was shown to contain an open reading frame of 954 nucleotides encoding a polypeptide of 317 amino acid residues with a calculated molecular mass of 33,872.

Another α -keto acid reductase, D-mandelate dehydrogenase, was purified from *Enterococcus faecalis* and designated as EfDMDH [78]. Its apparent molecular mass was 130,000 on gel filtration and 40,000 on SDS-PAGE. The enzyme had maximal activity at pH 4.5 and 35 to 45°C for the reduction of benzoylformic acid. EfDMDH had high activity for benzoylformate, phenylpyruvate, and 2-oxoisocaproate. The substrate specificity of EfDMDH was complementary to those of LoKAR1 and LmKAR1. Enzymatic properties and substrate specificity of α -keto acid reductases are summarized in [Table 25.6](#).

25.7 SYNTHESIS OF (*R*)- α -HYDROXY ACIDS WITH α -KETO ACID REDUCTASES

Various (*R*)- α -hydroxy acids were synthesized with whole-cell biocatalysts expressing an appropriate α -keto acid reductase for a target compound, as shown in [Figure 25.6](#). For LoKAR1, a coexpression plasmid—pSG-LOR1—was constructed which coexpressed BsGDH for the regeneration of NADPH. For EfDMDH and LmKAR1, coexpression plasmids—pSF-EFM2 and pSF-LMK1 respectively—with McFDH-26 were constructed for the regeneration of NADH.

Ethyl (*R*)-benzylactic acid is an intermediate for the synthesis of ACE inhibitors. It could be synthesized by several biological reduction processes: (1) asymmetric reduction of ethyl benzylpyruvate; (2) asymmetric reduction of benzylpyruvic acid followed by chemical esterification; (3) asymmetric reduction of 2-oxo-4-phenyl-3-butenic acid followed by chemical hydrogenation and esterification, as shown in [Figure 25.7](#). Ethyl benzylpyruvate could be reduced to ethyl (*R*)-benzylactate with some of our library enzymes, e.g., TdCR1 (a carbonyl reductase from *Torulaspora delbrueckii*). Benzylpyruvic acid and 2-oxo-4-phenyl-3-butenic acid, alternatively, could be reduced to (*R*)-benzylactic acid and (*R*)-2-hydroxy-4-phenyl-3-butenic acid, respectively, by LoKAR1. All enzymatic reductions were very effectively performed and the reductive process of benzylpyruvic acid with LoKAR1 was selected further because of productivity and easy purification of

TABLE 25.6

Properties of α -Keto Acid Reductases and α -Hydroxy Acid Dehydrogenases

Name	EfDMDH	LmKAR1	LoKAR1	LmDLDH	LcDHicDH
Origin	<i>Enterococcus faecalis</i>	<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>	<i>L. oenos</i>	<i>L. mesenteroides</i> subsp. <i>cremoris</i>	<i>Lactobacillus casei</i> subsp. <i>pseudo-plantarum</i>
E.C.	—	—	—	—	—
Cofactor	NADH	NADH	NADPH	NADH	NADH
Mr					
Native	130,000	62,600	46,000	80,000	74,000
Subunit	40,000	34,100	46,000	40,000	38,000
ORF	301 aa (33,100)	317 aa (33,900)	307 aa (33,049)	331 aa (36,316)	333 aa (36,893)
Optimum for reduction					
Temperature(°C)	35–45	50	40	ND	50
pH	4.5	5.5–6.0	6.5–7.0	ND	5.5–7.0
Activity					
Substrate	Benzoylformic acid	2-Chloro benzoylformic acid	Benzylpyruvic acid	Pyruvic acid	α -Ketoiso caproic acid
Specific activity (U/mg)	378	2,620	3.45	544	24.9
Substrate specificity (relative activity, %)					
Pyruvic acid	<1	<1	ND	100	ND
α -Ketoisocaproic acid	48.8	14.7	12.0	2.2	100
Benzoylformic acid	100	12.9	2.0	1.5	ND
2-Chloro-benzoylformic acid	23.7	100	<1	ND	ND
Phenylpyruvic acid	36.0	<1	12.0	3.2	251
Benzylpyruvic acid	<1	<1	100	ND	ND
Reversibility	Significantly irreversible	Irreversible	Irreversible	Reversible	Reversible
References	[78]	[77]	[75,76]	[79]	[80,81]

Mr, relative molecular mass; aa, amino acids; ND, not determined.

product. (*R*)-Benzylactic acid was synthesized from 2% benzylpyruvic acid and D-glucose with *E. coli* cells coexpressing LoKAR1 and BsGDH with 17.2 g/L concentration in >99% ee. By a similar process, (*R*)-2-hydroxy-5-phenylpentanoic acid and their derivatives could effectively be synthesized in >99% ee from their corresponding α -keto acids.

(*R*)-2-CMA is useful as an intermediate for the synthesis of several pharmaceuticals, such as an inhibitor of platelet aggregation and a reagent for diastereomeric resolution of several chiral amines. D-Lactate dehydrogenases from *Lactobacillus*, D-mandelate dehydrogenase

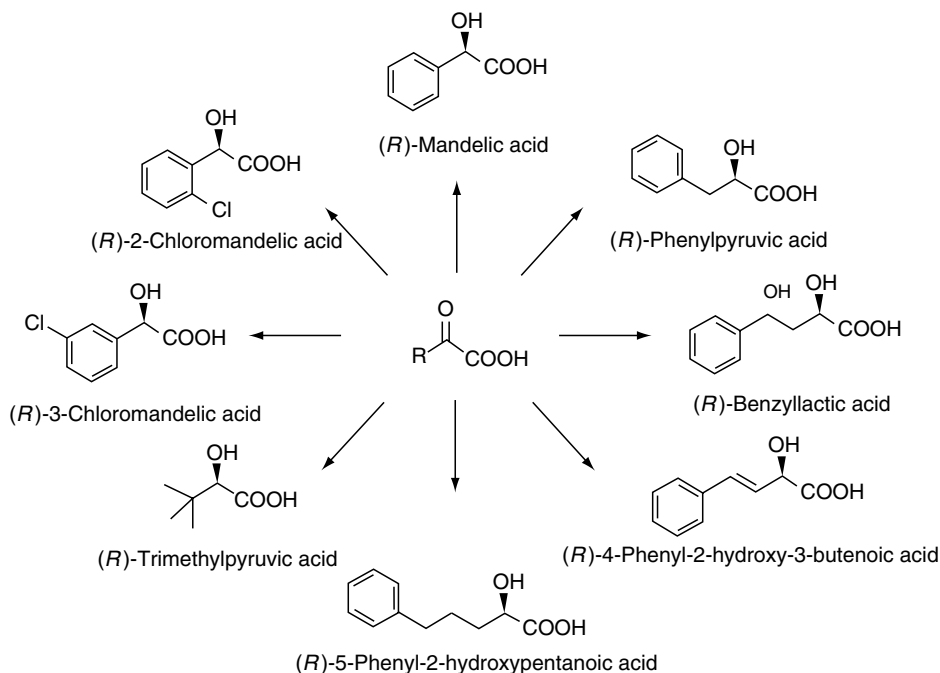


FIGURE 25.6 Synthesis of optically active α -hydroxy acids using several α -keto acid reductases.

such as EfDMDH, and LoKAR1 could not efficiently synthesize (R) -2-CMA from 2-chlorobenzoylformic acid because the substitution of chlorine on *o*-position, in general, decreased enzyme activity and stability. LmKAR1 is preferred to 2-chlorobenzoylformic acid as a substrate over benzoylformic acid and 3-chlorobenzoylformic acid, and is very efficiently synthesized (R) -2-CMA. The parent strain of LmKAR1, *L. mesenteroides* subsp. *dextranicum* synthesized (R) -2-CMA with 26.2 g/L in >99% ee. The reaction, however, required excess amount of glucose for the regeneration of NADPH and D-gluconate produced, as the by-product of the regeneration system caused an extra environmental burden.

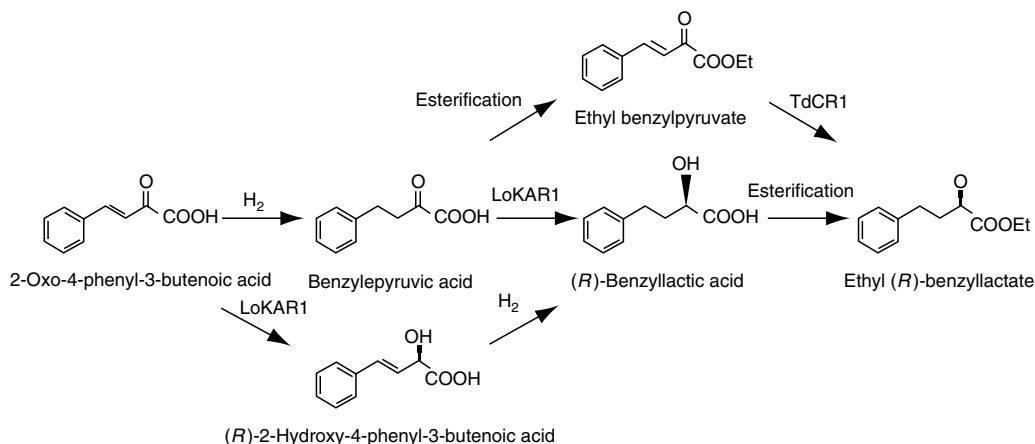


FIGURE 25.7 Synthetic routes of ethyl (R) -benzylsuccinate. LoKAR1, α -keto acid reductase from *Leuconostoc oenos*. TdCR1, carbonyl reductase from *Torulaspora delbrueckii*.

The use of recombinant *E. coli* cells coexpressing LmKAR1 and McFDH-26 as a biocatalyst enabled more efficient production of (*R*)-2-CMA with 68.0 g/L in >99% ee from 7% 2-chlorobenzoylformic acid, and formic acid with carbon dioxide as a by-product caused lesser environmental burden. By a similar process, (*R*)-trimethylsuccinic acid was synthesized quantitatively from 5% trimethylpyruvic acid in >99% ee.

(*R*)-Phenyllactic acid and D-leucic acid [(*R*)-2-hydroxyisocaproic acid] are also useful as raw materials and intermediates for the synthesis of pharmaceuticals. These compounds and their derivatives were efficiently synthesized with *E. coli* cells coexpressing EfDMDH and McFDH-26. As described earlier, our α -keto acid reductases library having distinct substrate specificity enabled extremely broad application in reduction processes.

25.8 ROBUST NADH REGENERATOR, McFDH-26

Biological asymmetric reduction requires an efficient regeneration system of the coenzyme NAD(P)H, especially in recombinant *E. coli* cells that highly express ADHs and carbonyl reductases, as *E. coli* cells have poor coenzyme-regeneration activities [11,82]. To overcome this problem, a GDH from *B. megaterium* as an NADPH-regeneration system was coexpressed in *E. coli* with an aldehyde reductase from *S. salmonicolor* [9]. Currently, the coenzyme-regeneration method using GDH is the most frequently used because it can regenerate not only NADPH but also NADH, and has high activity in broad pH range as well as high stability against several organic compounds. However, GDHs have several problems: gluconate is generated as a by-product in an amount equimolar to the desired optically active alcohol, which interferes with purification and is not environment-friendly.

NAD⁺-dependent FDHs (E.C. 1.2.1.2), have also been used for the regeneration of NADH *in vitro* [83]. The coenzyme-regeneration method using FDH has several advantages: (1) its reaction is irreversible; (2) formic acid as its substrate is a small molecule and is inexpensive; (3) carbon dioxide as its product does not inhibit and inactivate carbonyl reductases involved and is easily removed from a target product; and (4) its environmental burden is relatively low. FDH, however, has a low activity and is labile to heat, several metals, oxidation by air, and hydrophobic compounds, especially α -haloketones.

Using an FDH from *M. vaccae* [30], which was one of the highest specific-activity and stable FDHs reported previously, we compared FDH with GDH as an NADH regenerator to produce (*S*)-ECHB from ECAA with *E. coli* cells expressing KaCR1 [59]. Using BsGDH, the amount of ECHB reached 45.6 g/L in >99% ee (*S*), as shown in Figure 25.8. The use of BsGDH yielded 45.6 g/L of (*S*)-ECHB from 50 g/L of ECAA, but the use of McFDH yielded

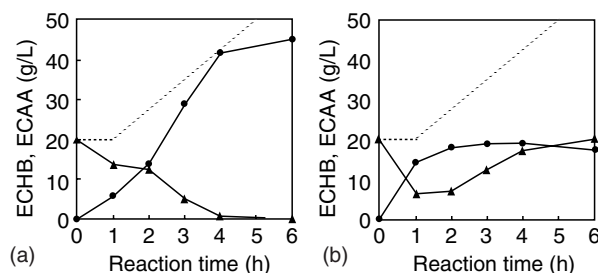


FIGURE 25.8 Synthesis of (*S*)-ECHB with *E. coli* cells harboring pSG-KAR1 and pSFR415. (a) pSG-KAR1, a coexpression plasmid of KaCR1 and BsGDH; (b) pSFR415, a coexpression plasmid of KaCR1 and McFDH. Symbols: ●, ECHB; ▲, ECAA. The total amount of ECAA added is shown by a dotted line.

only 19.0 g/L of ECHB in >99% ee (*S*). The low productivity in the case of FDH was suggested to result from the low activity and instability of FDH against ECAA.

On the assumption that the modification of some Cys residues by ECAA caused the inactivation of FDH, we examined the substitution of five Cys residues by site-directed mutagenesis (Cys-6, -146, -249, -256, and -355) among seven Cys residues that were not conserved in several FDHs [84]. Mutant FDHs were evaluated by enzyme activity and amounts of (*S*)-ECHB produced from 3% ECAA with *E. coli* cells coexpressing the KaCR1 gene without NAD⁺, which had a protective effect against the inactivation by ECAA. C146S and C256S mutants yielded a higher amount (19.3 g/L and 30.5 g/L, respectively) of (*S*)-ECHB than wild-type FDH (17.5 g/L). Cys-146 mutations (C146S and C6S/C146S/C256S) did not show as remarkable effects as Cys-256 mutations, but yielded larger amounts of (*S*)-ECHB in spite of lower FDH activity. Cys-6, Cys-146, and Cys-256 residues were substituted by Ala, Ser, and Val in several combinations, and in consequence a C6A/C146S/C256V mutant, designated as McFDH-26, was selected as the best, as shown in Table 25.7. *E. coli* cells coexpressing an McFDH-26 gene and a KaCR1 gene had 8.15 U/mL-cultured broth of FDH activity and produced 32.2 g/L of (*S*)-ECHB from 30 g/L of ECAA. FDH mutants were characterized from the viewpoint of the tolerance to ECAA. Substitutions of Cys-256 by Ala, Ser, and Val gave higher tolerance to ECAA, as shown in Table 25.8. In the course of experiments on the inactivation of the enzyme, ECAA, ethyl acetoacetate, and ethyl acetate were found to activate FDH, though some mutant FDHs were inactivated more or less by incubation with ECAA for 20 min. C146S and C146A mutants, especially, were activated to 140 to 220%, while FDHs that had Cys-146 were activated only slightly to 100 to 120%. These findings strongly suggested that Cys-256 mutations caused the tolerance against ECAA, and Cys-146 mutations caused the activation by several organic compounds. McFDH-26 was evaluated from the productivity of (*S*)-ECHB on a mini-jar scale. Using *E. coli* cells coexpressing McFDH-26 (C6A/C146S/C256V) and KaCR1, the amount of ECHB reached 49.1 g/L in >99% ee (*S*), as shown in Figure 25.9. These results showed that McFDH-26 had comparative capacity to GDH as an NADH regenerator. Recently, an α -haloketone-resistant formate dehydrogenase (TsFDH) was obtained from *Thiobacillus* sp.

TABLE 25.7
Effects of Substitutions of Cys-Residues by Ala, Ser, and Val in McFDH

ORF No.	Amino Acid Residues			FDH (U/mg)	(<i>S</i>)-ECHB (g/L)
	6-Cys	146-Cys	256-Cys		
Wild	—	—	—	3.24	17.5
11	Ser	—	Ser	0.285	15.9
16	Ala	—	Ser	1.98	25.8
17	Val	—	Ser	1.62	25.2
18	Ser	—	Ala	0.635	22.4
19	Ser	—	Val	0.504	25.5
25	Ala	—	Val	1.83	29.6
23	—	—	Val	2.58	30.6
24	—	Ser	Val	2.29	31.0
26	Ala	Ser	Val	2.65	32.2
27	Ala	Ala	Val	1.15	27.7
28	Ala	Val	Val	1.49	26.9

(*S*)-ECHB, the amount of (*S*)-ECHB synthesized from 30 g/L ECAA with each recombinant whole-cell biocatalyst without NAD⁺.

TABLE 25.8

Effects of Mutant FDHs on the Resistance against ECAA and the Activation by Ethyl Acetate

ORF No.	6-Cys	146-Cys	256-Cys	(S)-ECHB ^a (g/L)	Activation ^b by AcOEt (%)	Tolerance ^c against ECAA (%)
15	—	—	—	17.5	121	7.43
9	Ser	—	—	9.4	ND	6.58
23	—	—	Val	30.6	111	100
11	Ser	—	Ser	15.9	105	125
16	Ala	—	Ser	25.8	109	97.4
17	Val	—	Ser	25.2	105	92.1
18	Ser	—	Ala	22.4	125	120
19	Ser	—	Val	25.5	110	94.0
21	—	—	Ser	29.1	125	99.3
20	—	Ser	—	19.3	181	7.67
12	Ser	Ser	Ser	24.5	225	116
22	—	Ser	Ser	25.9	224	118
24	—	Ser	Val	31.0	185	104
25	Ala	—	Val	29.6	111	94.5
26	Ala	Ser	Val	32.2	137	108
27	Ala	Ala	Val	27.7	219	106
28	Ala	Val	Val	26.9	84.5	100

^a(S)-ECHB was synthesized from 3% ECAA without NAD⁺ using whole cells in a flask.^bFDH was assayed with 5% AcOEt. To calculate the relative activity, the activity without AcOEt was taken as 100%.^cFDH was assayed after the incubation for 20 min with 20 mM ECAA at 25°C. To calculate the relative activity, the activity without incubation was taken as 100%.

strain KNK65MA by screening [85]. The corresponding residue of TsFDH to 256-Cys of McFDH is 256-Val, similarly to McFDH-26, and 256-Val of TsFDH was thought to cause the resistance of TsFDH against α -haloketone. 145-Cys residue of PsFDH corresponding to 146-Cys of McFDH was reported to be located in the region of the intersubunit contact [86] and not modified by DTNB even in the presence of 8 M urea to dissociate the enzyme into individual subunits [87]. C146A, C146S, and C146V mutations may strengthen an intersubunit binding. The relationship between this speculation and the activation by organic solvent remains to be unclear. The tolerance to organic solvent and activation of enzyme by organic solvent is very useful for bioconversion, because most artificial substrates for pharmaceutical intermediates are hydrophobic.

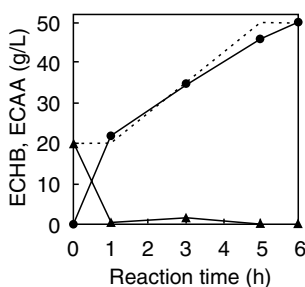


FIGURE 25.9 Synthesis of (S)-ECHB with *E. coli* cells coexpressing KaCR1 and McFDH-26. Symbols: ●, ECHB; ▲, ECAA. The total amount of ECAA added is shown by a dotted line.

25.9 REMARKS

The cloned enzyme library has several advantages: (1) highly active and regio- and stereo-selective; (2) speedy enzyme screening because their characteristics, such as substrate specificity and stereoselectivity, are well known; (3) easy scale-up of process because their host is common *E. coli* and culture, induction, bioconversion, and purification methods may be quite similar; (4) safe and green (white) process as the host *E. coli* K-12 strain is nonpathogenic to humans and animals, and is not viable in the environment. We have constructed a cloned enzyme library comprising 140 unique enzymes having different substrate specificity and enantioselectivity, and 70 ADHs and carbonyl reductases. Conventional enzyme screening has been significantly replaced by enzyme library screening at our company. Not only conventional screening but also the use of environmental DNA screening will continue to be necessary for broader applicability of biocatalytic processes and enrichment of the enzyme library. As shown in this chapter, an McFDH mutant, McFDH-26, has solved some of the problems associated with NADH cofactor regeneration. Incapability of NADPH regeneration by FDH, however, remains to be solved on an industrial scale. A biphasic system, such as *n*-butyl acetate and water, improved enzyme stability, solubility of substrates and products, and space yields [88,89]. These innovations have increased the number of industrial manufacturing by asymmetric reduction. For continuous improvement in commercial production of chiral compounds by asymmetric reduction, some innovations are necessary. One is an artificial design of a biocatalyst including the alteration of substrate specificity and some properties of enzymes by directed evolution and also engineering of an expression host. Improvement in process engineering and downstream processing is required as the regulation of impurities in a product has become increasingly important in the pharmaceutical application.

REFERENCES

1. Hollingsworth, R.I. and Wang, G., General three carbon chiral synthons from carbohydrates: chiral pool and chiral auxiliary approaches, *ACS Symp. Ser.*, 841, 85, 2003.
2. Yamada, S. et al., Combined effect of polymorphism and process on preferential crystallization: example with (\pm) -5-(4'-methylphenyl)-5-methylhydantoin, *Tetrahedron Asymmetry*, 9, 1713, 1998.
3. Kinbara, K. et al., Effect of a substituent on an aromatic group in diastereomeric resolution, *Tetrahedron*, 56, 6651, 2000.
4. Ghanem, A. and Aboul-Enein, H.Y., Application of lipases in kinetic resolution of racemates, *Chirality*, 17, 1, 2004.
5. Franco, P. et al., The impact of productivity in the preparative separation of enantiomers by chromatography, *Chimica Oggi*, 22, 28, 2004.
6. Camps, P. and Munoz-Torrero, D., Synthesis and applications of (*R*)- and (*S*)-pantolactone as chiral auxiliaries, *Curr. Org. Chem.*, 8, 1339, 2004.
7. Ohkuma, T. and Noyori, R., *Comprehensive Asymmetric Catalysis*, Suppl. 1, Springer-Verlag, Berlin, 2004, p. 1.
8. Patel, R.N., Microbial/enzymatic synthesis of chiral intermediates for pharmaceuticals, *Enzyme Microb. Technol.*, 31, 804, 2002.
9. Kataoka, M. et al., Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by *Escherichia coli* transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes, *Appl. Microbiol. Biotechnol.*, 51, 486, 1999.
10. Galkin, A. et al., Synthesis of optically active amino acids from α -keto acids with *Escherichia coli* cells expressing heterologous genes, *Appl. Environ. Microbiol.*, 63, 4651, 1997.
11. Yamamoto, H., Matsuyama, A., and Kobayashi, Y., Synthesis of ethyl (*R*)-4-chloro-3-hydroxybutanoate with recombinant *Escherichia coli* cells expressing (*S*)-specific secondary alcohol dehydrogenase, *Biosci. Biotechnol. Biochem.*, 66, 481, 2002.

12. Tanaka, R., Iwata, H., and Ishiguro, M., Synthesis of 5,6-*cis*-penems, *J. Antibiot.*, 43, 1608, 1990.
13. Nakatsuka, T. et al., A facile conversion of the phenylthio group to acetoxy by copper reagents for a practical synthesis of 4-acetoxyazetidin-2-one derivatives from (*R*)-butane-1,3-diol, *J. Chem. Soc. Chem. Commun.*, 662, 1991.
14. Hart, D.J. and Ha, D.C., An enantioselective approach to carbapenem antibiotics: formal synthesis of (+)-thienamycin, *Tetrahedron Lett.*, 26, 5493, 1985.
15. Matsuyama, A., Kobayashi, Y., and Ohnishi, H., Microbial production of optically active 1,3-butanediol from 4-hydroxy-2-butanone, *Biosci. Biotech. Biochem.*, 57, 348, 1993.
16. Matsuyama, A., Kobayashi, Y., and Ohnishi, H., Microbial production of optically active 1,3-butanediol from the racemate, *Biosci. Biotechnol. Biochem.*, 57, 685, 1993.
17. Yamamoto, H. et al., Purification and characterization of (*S*)-1,3-butanediol dehydrogenase from *Candida parapsilosis*, *Biosci. Biotechnol. Biochem.*, 59, 1769, 1995.
18. Yamamoto, H. et al., Cloning and expression in *Escherichia coli* of a gene coding for a secondary alcohol dehydrogenase from *Candida parapsilosis*, *Biosci. Biotechnol. Biochem.*, 63, 1051, 1999.
19. Reid, M.F. and Fewson, C.A., Molecular characterization of microbial alcohol dehydrogenases, *Crit. Rev. Microbiol.*, 20, 13, 1994.
20. Peters, J., Minuch, T., and Kula, M.-R., A novel NADH-dependent carbonyl reductase with an extremely broad substrate range from *Candida parapsilosis*: purification and characterization, *Enzyme Microb. Technol.*, 15, 950, 1993.
21. Itoh, N. et al., Purification and characterization of phenylacetaldehyde reductase from a styrene-assimilating *Corynebacterium* strain ST-10, *Appl. Environ. Microbiol.*, 63, 3783, 1997.
22. Wang, J.-C. et al., Cloning, sequence analysis, and expression in *Escherichia coli* of the gene encoding phenylacetaldehyde reductase from styrene-assimilating *Corynebacterium* sp. strain ST-10, *Appl. Microbiol. Biotechnol.*, 52, 386, 1999.
23. Xie, S.-X., Ogawa, J., and Shimizu, S., NAD⁺-dependent (*S*)-specific secondary alcohol dehydrogenase involved in stereoinversion of 3-pentyn-2-ol catalyzed by *Nocardia fusca* AKU 2123, *Biosci. Biotech. Biochem.*, 63, 1721, 1999.
24. Hummel, W., New alcohol dehydrogenases for the synthesis of chiral compounds, *Adv. Appl. Microbiol.*, 58, 145, 1997.
25. Abokitsel, K. and Hummel, W., Cloning, sequence analysis, and heterologous expression of the gene encoding an (*S*)-specific alcohol dehydrogenase from *Rhodococcus etythropolis* DSM 43297, *Appl. Microbiol. Biotechnol.*, 62, 380, 2003.
26. Stampfer, W. et al., Biocatalytic asymmetric hydrogen transfer, *Angew. Chem. Int. Ed. Eng.*, 41, 1014, 2002.
27. Yamamoto, H., Matsuyama, A., and Kobayashi, Y., Synthesis of (*R*)-1,3-butanediol by enantioselective oxidation using whole recombinant *Escherichia coli* cells expressing (*S*)-specific secondary alcohol dehydrogenase, *Biosci. Biotechnol. Biochem.*, 66, 925, 2002.
28. Anraku, Y. and Gennis, R.B., The aerobic respiratory chain of *Escherichia coli*, *Trends Biochem. Sci.*, 12, 262, 1987.
29. Kasahara, M. and Anraku, Y., Succinate- and NADH oxidase systems of *Escherichia coli* membrane vesicles: mechanism of selective inhibition of the systems by zinc ions, *J. Biochem.*, 76, 967, 1974.
30. Karzanov, V.V. et al., An alternative NAD⁺-dependent formate dehydrogenases in the facultative methylotroph *Mycobacterium vaccae* 10, *FEMS Microbiol. Lett.*, 81, 95, 1991.
31. Galkin, A. et al., Cloning of formate dehydrogenase gene from a methanol-utilizing bacterium *Mycobacterium vaccae* N10, *Appl. Microbiol. Biotechnol.*, 44, 479, 1995.
32. Zhou, B. et al., Stereochemical control of yeast reductions. 1. Asymmetric synthesis of L-carnitine, *J. Am. Chem. Soc.*, 105, 5925, 1983.
33. Kitamura, M. et al., A Practical asymmetric synthesis of carnitine, *Tetrahedron Lett.*, 29, 1555, 1988.
34. Jung, M.E. and Shaw, T.J., Total synthesis of (*R*)-glycerol acetone and the antiepileptic and hypotensive drug (–)- γ -amino- β -hydroxybutyric acid (GABOB): use of vitamin C as a chiral starting material, *J. Am. Chem. Soc.*, 102, 6304, 1980.

35. Pifferi, G. and Pinza, M., Cyclic GABA-GABOB analogues. I. Synthesis of new 4-hydroxy-2-pyrrolidone derivatives, *Farmaco. Ed. Sci.*, 32, 602, 1977.
36. Karanewsky, D.S. et al., Phosphorous-containing inhibitors of HMG-CoA reductase. 1. 4-[2-(arylethyl)hydroxyphosphinyl]-3-hydroxy-butanoic acids: a new class of cell-sensitive inhibitors of cholesterol biosynthesis, *J. Med. Chem.*, 33, 2925, 1990.
37. Santaniello, E., Casati, R., and Milani, F., Chiral synthesis of a component of *Amanita muscaria*, (–)-4-hydroxypyrrolidin-2-one, and assessment of its absolute configuration, *J. Chem. Res. (Synops)*, 132, 1984.
38. Shieh, W., Gopalan, A.S., and Sih, C.J., Stereochemical control of yeast reductions. 5. Characterization of the oxidoreductases involved in the reduction of β -keto esters, *J. Am. Chem. Soc.*, 107, 2993, 1985.
39. Patel, R.N. et al., Stereoselective reduction of β -keto esters by *Geotrichum candidum*, *Enzyme Microb. Technol.*, 14, 731, 1992.
40. Kita, K. et al., Purification and characterization of new aldehyde reductases from *Sporobolomyces salmonicolor* AKU4429, *J. Mol. Catal. B Enzym.*, 6, 305, 1999.
41. Kataoka, M. et al., A novel NADPH-dependent carbonyl reductase of *Candida macedoniensis*: purification and characterization, *Arch. Biochem. Biophys.*, 294, 469, 1992.
42. Wada, M. et al., Purification and characterization of NADPH-dependent carbonyl reductase, involved in stereoselective reduction of ethyl 4-chloro-3-oxobutanoate, from *Candida magnoliae*, *Biosci. Biotech. Biochem.*, 62, 280, 1998.
43. Yamamoto, H. et al., Purification and properties of a carbonyl reductase that is useful for the production of ethyl (S)-4-chloro-3-hydroxybutanoate from *Kluyveromyces lactis*, *Biosci. Biotechnol. Biochem.*, 66, 1775, 2002.
44. Stoops, J.K. et al., Studies on the yeast fatty acid synthetase, *J. Biol. Chem.*, 253, 4464, 1978.
45. Wakil, S.J., Stoops, J.K., and Joshi, V.C., Fatty acid synthesis and its regulation, *Ann. Rev. Biochem.*, 52, 537, 1983.
46. Toomey, R.E. and Walkil, S.J., Studies on the mechanism of fatty acid synthesis. XV. Preparation and general properties of β -ketoacyl acyl carrier protein reductase from *Escherichia coli*, *Biochim. Biophys. Acta*, 116, 189, 1966.
47. Rawlings, M. and Cronan, J.E. Jr., The gene encoding *Escherichia coli* acyl carrier protein lies within a cluster of fatty acid biosynthetic genes, *J. Biol. Chem.*, 267, 5751, 1992.
48. Schubert, P., Steinbuchel, A., and Schlegel, H.G., Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly- β -hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*, *J. Bacteriol.*, 170, 5837, 1988.
49. Vagelos, P.R., Alberts, A.W., and Majerus, P.W., β -Ketoacyl acyl carrier protein reductase. *Meth. Enzymol.*, 14, 60, 1969.
50. Morbidoni, H.R., de Mendoza, D., and Cronan, J.E. Jr., *Bacillus subtilis* acyl carrier protein is encoded in a cluster of lipid biosynthesis genes, *J. Bacteriol.*, 178, 4794, 1996.
51. Haywood, G.W. et al., The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism *Alcaligenes eutrophus*, *FEMS Microbiol. Lett.*, 52, 259, 1988.
52. Peoples, O.P. and Sinskey, A.J., Poly- β -hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16, *J. Biol. Chem.*, 264, 15293, 1989.
53. Fukui, T. et al., Purification and characterization of NADP-linked acetoacetyl-CoA reductase from *Zoogloea ramigera* I-16-M, *Biochim. Biophys. Acta*, 917, 365, 1987.
54. Peoples, O.P. and Sinskey, A.J., Fine structural analysis of the *Zoogloea ramigera* *phbA-phbB* locus encoding β -ketothioase and acetoacetyl-CoA reductase: nucleotide sequence of *phbB*, *Mol. Microbiol.*, 3, 349, 1989.
55. Yamamoto, H., Matsuyama, A., and Kobayashi, Y., Synthesis of ethyl (S)-4-chloro-3-hydroxybutanoate using *fabG*-homologues, *Appl. Microbiol. Biotechnol.*, 61, 133, 2003.
56. Lampel, K.A. et al., Characterization of the developmentally regulated *Bacillus subtilis* glucose dehydrogenase gene, *J. Bacteriol.*, 166, 238, 1986.
57. Takebe, I. and Kitahara, K., Levels of nicotinamide nucleotide coenzymes in lactic acid bacteria, *J. Gen. Appl. Microbiol.*, 9, 31, 1963.

58. London, J. and Knight, M., Concentration of nicotinamide nucleotide coenzymes in micro-organism, *J. Gen. Microbiol.*, 44, 241, 1966.
59. Yamamoto, H. et al., A novel NADH-dependent carbonyl reductase from *Kluyveromyces aestuarii* and comparison of NADH-regeneration systems for the synthesis of ethyl (S)-4-chloro-3-hydroxybutanoate, *Biosci. Biotechnol. Biochem.*, 68, 638, 2004.
60. Kudoh, M. and Yamamoto, H., (R)-2-Octanol dehydrogenase, process for producing the enzyme, DNA encoding the enzyme and process for producing alcohol by using the same, WO Patent Appl. 2001061014, 2001.
61. Yamamoto, H. et al., Robust NADH-regenerator: improved α -haloketone-resistant formate dehydrogenase, *Appl. Microbiol. Biotechnol.*, 67, 33, 2005.
62. Urbach, H. and Henning, R., A favourable diastereoselective synthesis of N-(1-S-ethoxycarbonyl-3-phenylpropyl)-9S-alanine, *Tetrahedron Lett.*, 25, 1143, 1984.
63. Boyer, S.K. et al., Note on the synthesis of an optically active ACE inhibitor with amino-oxo-benzazepine-1-alkanoic-acid structure by means of an enantioconvergent crystallization-based resolution, *Helv. Chim. Acta*, 71, 337, 1988.
64. Sasaki, T. et al., A new anthelmintic cyclodepsipeptide, PF1022A, *J. Antibiot.*, 45, 692, 1992.
65. Badore, A. and Frehel, D., Dextro-rotatory enantiomer of methyl α -5 (4,5,6,7-tetrahydro(3,2-c)thieno pyridyl) (2-chlorophenyl)-acetate and the pharmaceutical compositions containing it, U.S. Patent Appl. 4,847,265, 1989.
66. Seo, A., Hiraga, K., and Omi, T., Preparation of optically active ketene dithioketal derivative and its use as medicinal fungicide (in Japanese), Jap. Patent Appl. 02-275877, 1990.
67. Biquard, D., Absorption of certain classes of organic molecules, *Ann. Chim.*, 20, 97, 1933.
68. Collet, A. and Jacques, J., Optical antipode mixtures. V. Substituted mandelic acids, *Bull. Soc. Chim. France*, 12, 3330, 1973.
69. Valente, E. et al., Discrimination in resolving systems. II. Ephedrine-substituted mandelic acids, *Chirality*, 7, 652, 1995.
70. Yamamoto, K. et al., Production of R-(–)-mandelic acid from mandelonitrile by *Alcaligenes faecalis* ATCC 8750, *Appl. Environ. Microbiol.*, 57, 3028, 1991.
71. Glieder, A. et al., Comprehensive step-by-step engineering of an (R)-hydroxynitrile lyase for large-scale asymmetric synthesis, *Angew. Chem. Int. Ed. Eng.*, 42, 4815, 2003.
72. Simon, E.S., Plante, R., and Whitesides, G.M., D-Lactate dehydrogenase substrate specificity and use as a catalyst in the synthesis of homochiral 2-hydroxy acids, *Appl. Biochem. Biotechnol.*, 22, 169, 1989.
73. Hummel, W., Schutte, H., and Kula, M.R., Large-scale production of D-lactate dehydrogenase for the stereospecific reduction of pyruvate and phenylpyruvate, *Eur. J. Appl. Microbiol. Biotechnol.*, 18, 75, 1983.
74. Hummel, W. et al., New NADH-dependent dehydrogenases from *Lactobacillus* strains for synthesis and analysis L- and D-hydroxyisocaproate dehydrogenase, D-mandelate dehydrogenase and (+)-acetoin dehydrogenase large-scale purification, *Biocatalysis*, 4, 79–80, 1990.
75. Nakajima, N. et al., Purification and characterization of aldehyde reductase from *Leuconostoc dextranum*, *Biosci. Biotech. Biochem.*, 57, 160, 1993.
76. Nakajima, T. et al., unpublished data, 2005.
77. Yamamoto, H. and Kimoto, N., α -Keto acid reductase, method for producing the same, and method for producing optically active α -hydroxy acids using the same, U.S. Patent Appl. 2004086993, 2004.
78. Tamura, Y. et al., Two forms of NAD-dependent D-mandelate dehydrogenase in *Enterococcus faecalis* IAM 10071, *Appl. Environ. Microbiol.*, 68, 947, 2002.
79. Dartois, V. et al., Purification, properties and DNA sequence of the D-lactate dehydrogenase from *Leuconostoc mesenteroides* subsp. *cremoris*, *Res. Microbiol.*, 146, 291, 1995.
80. Hummel, W., Schutte, H., and Kula, M.R., D-2-Hydroxyisocaproate dehydrogenase from *Lactobacillus casei*: a new enzyme suitable for stereospecific reduction of 2-ketocarboxylic acids, *Appl. Microbiol. Biotechnol.*, 21, 7, 1985.
81. Lerch, H.P. et al., Cloning, sequencing and expression in *Escherichia coli* of D-2-hydroxyisocaproate dehydrogenase gene of *Lactobacillus casei*, *Gene*, 78, 47, 1989.

82. Kataoka, M. et al., Enzymatic production of ethyl (*R*)-4-chloro-3-hydroxybutanoate: asymmetric reduction of ethyl 4-chloro-3-oxobutanoate by an *Escherichia coli* transformant expressing the aldehyde reductase gene from yeast, *Appl. Microbiol. Biotechnol.*, 48, 699, 1997.
83. Wichmann, R. et al., Continuous enzymatic transformation in an enzyme membrane reactor with simultaneous NAD(H) regeneration, *Biotechnol. Bioeng.*, 23, 2789, 1981.
84. Popov, V.O. and Lamzin, V.S., NAD⁺-dependent formate dehydrogenase, *Biochem. J.*, 301, 625, 1994.
85. Nanba, H., Takaoka, Y., and Hasegawa, J., Purification and characterization of an α -haloketone-resistant formate dehydrogenase from *Thiobacillus* sp. strain KNK65MA, and cloning of the gene, *Biosci. Biotech. Biochem.*, 67, 2145, 2003.
86. Lamzin, V.S. et al., Crystal structure of NAD-dependent formate dehydrogenase, *Eur. J. Biochem.*, 206, 441, 1992.
87. Fedorchuk, V.V. et al., Effect of interactions between amino acid residues 43 and 61 of thermal stability of bacterial formate dehydrogenases, *Biochemistry* (Moscow), 67, 1385, 2002.
88. Shimizu, S. et al., Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by a microbial aldehyde reductase in an organic solvent–water biphasic system, *Appl. Environ. Microbiol.*, 56, 2374, 1990.
89. Kizaki, N. et al., Synthesis of optically pure ethyl (*S*)-4-chloro-3-hydroxybutanoate by *Escherichia coli* transformant cells coexpressing the carbonyl reductase and glucose dehydrogenase genes, *Appl. Microbiol. Biotechnol.*, 55, 590, 2001.

26 Comparative Analysis of Chemical and Biocatalytic Syntheses of Drug Intermediates

Michael J. Homann, Wen-Chen Suen, Ningyan Zhang, and Aleksey Zaks

CONTENTS

26.1	Introduction	645
26.2	Process Development	646
26.2.1	Posaconazole	646
26.2.2	Ribavarin Alanine Ester	648
26.2.3	NK ₁ /NK ₂ Receptor Antagonist	651
26.3	Directed Evolution to Improve the Functional Properties of CALB.....	652
26.3.1	Application of Error-Prone PCR for Improving Thermostability	653
26.3.2	Application of DNA Family Shuffling for Improving Activity	653
26.4	Production of Chimeric Lipase B by Recombinant Fermentation.....	655
26.5	Conclusions	656
	Acknowledgments	657
	References	657

26.1 INTRODUCTION

In the last decade, biocatalysis has been firmly established as a powerful synthetic tool. While the majority of synthetic targets can be attained by purely chemical means, chemistry alone often does not provide the most elegant solutions. Numerous examples of biosynthetic approaches incorporating chemistry and biology have resulted in a significant simplification of synthetic strategies to generate complex chiral molecules including drugs and drug intermediates. Indeed, the number of biocatalytic approaches to chiral intermediates has grown significantly in recent years in support of a continuously expanding market for single enantiomer drugs [1,2]. Despite numerous examples of integrated biochemical approaches reported in the literature, some argue that the area of biocatalysis has not fulfilled earlier expectations based on the limited number of commercial biological processes. However, this position overlooks the fact that, while large-scale commercial bioprocesses garner most of the attention, they represent only a fraction of the overall contribution of biocatalysis to drug discovery and development.

Today biocatalysts are commonly employed in initial discovery-type approaches to biologically active compounds and in creating targeted libraries [3–6]. They are also widely used for generation of metabolites in support of drug safety and metabolism programs [7–12] and continue to play a critical role in the area of process development. Enzyme-based approaches are routinely incorporated into initial drug supply routes due to their simplicity as they rarely require protection or deprotection steps and often enable rapid access to kilogram quantities of drug substance or key intermediates. While the cost of these initial routes is rarely critical, the selection criterion for later-stage syntheses shifts from timely access to drug supplies, to providing a reliable, safe, and economical process. Hence, the definition of the “best” process depends largely on the stage of the drug development program and, consequently, on the selection criteria. As a drug candidate progresses from early development into commercialization the initial synthetic route is often modified or completely redesigned to satisfy the commercial process needs. Therefore, as the commercial process evolves, biocatalytic steps may be incorporated, modified, or removed altogether.

In this review, we will describe three case studies involving development of drug candidates at Schering-Plough that at one point or another utilized a biocatalytic approach. We will describe criteria for selecting a particular approach as it relates to a specific stage of development of the drug candidate. We will also present examples of new strategies for enhancing the functional properties of biological catalysts that should ultimately promote their synthetic utility.

26.2 PROCESS DEVELOPMENT

26.2.1 POSACONAZOLE

Posaconazole is a broad-spectrum orally active azole-antifungal discovered and developed at Schering-Plough (Figure 26.1). This compound exhibits enhanced activity against systemic *Candida* and *Aspergillus* infections, thus providing greater potency for resolving fungal infections when compared with standard therapies. Development of this antifungal represents a case in which an initial chemical approach designed to provide speedy delivery of the drug substance for phase I clinical studies was later replaced with a new route that included a biocatalytic step. This second generation synthesis being significantly more efficient than the original one was successfully scaled up to a commercial process.

The initial supply route to the key 2*R*, 4*S*-phenylsulfonate intermediate, **8**, used Sharpless epoxidation to form a chiral epoxy alcohol **2** [13,14] (Figure 26.1). Although a pilot plant campaign based on this approach generated 10 kg of drug substance to support initial clinical trials, the route was deemed inadequate for scale-up. Indeed, the synthesis was long and provided poor diastereomeric control in cyclization, resulting in predominant formation (3:2) of *trans*-**8**, and thus required chromatography to separate the isomers. Clearly, a better approach was needed to support increasing drug substance requirements.

Further process improvement efforts resulted in the discovery of a highly efficient one-step three-component coupling giving the desired *cis*-triazole, albeit in the racemic form. It was thought that the desired stereochemistry at the 4-position of the tetrahydrofuran ring of the intermediate **15** (Figure 26.2) could be achieved by selectively blocking one of the two primary hydroxyls of the diol **12** by enzymatic transesterification. To that end, about 200 enzymes were screened for selective acylation of the diol in various organic solvents with a number of acyl donors. *Candida antarctica* lipase B (CALB) showed the highest selectivity for the pro-*S* acylation of **12**. Following reaction optimization including examination of combinations of solvents, acylating agents, temperatures, substrate concentrations, and additives, the first-generation biocatalytic step was established [15,16] (Table 26.1).

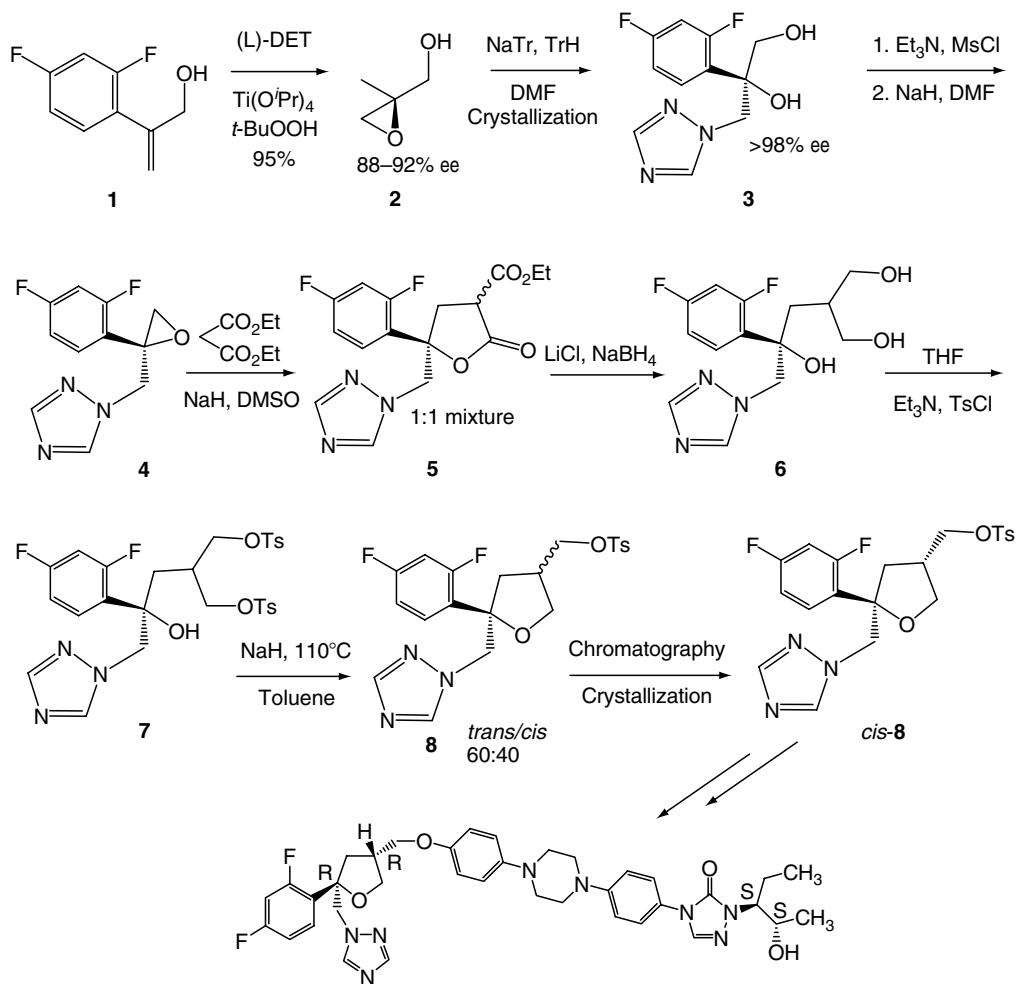


FIGURE 26.1 Initial chemical route to posaconazole.

Although >100 kg of **13a** were generated under conditions described in Table 26.1 the outcome of the biocatalytic step utilizing vinyl acetate as an acylating agent was somewhat unpredictable. The yield of monoester product after reaching 80 to 85% decreased to 70 to 75% if the reaction was allowed to progress. Moreover, both the enantiomeric purity of **13a** and the level of the diacetate by-product varied from batch to batch. Since lipase-catalyzed esterifications proceed through the formation of an acyl–enzyme intermediate [17], it was hypothesized that an acylating agent with a steric volume larger than vinyl acetate would increase the steric hindrance within the enzyme's active site. This was expected to decrease the rate of acyl transfer to the undesired pro-*R* hydroxyl, thereby improving the enzyme's enantioselectivity. To test this hypothesis, 11 acylating agents were examined in 8 solvents to determine an optimal solvent–acylating agent combination. As predicted, isobutyric anhydride was found to be a significantly superior acylating agent, reducing the maximum level of diester formation to no more than 7%. To minimize the possibility of nonenzymatic acylation with a highly reactive anhydride, the reaction temperature was lowered to -15°C . Lowering reaction temperature and introducing solid NaHCO_3 also eliminated the risk of

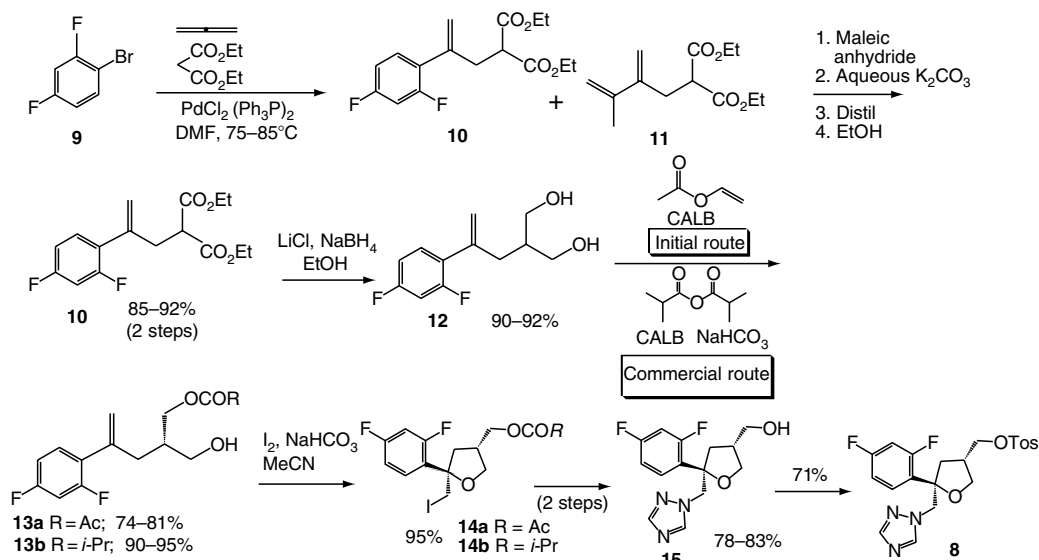


FIGURE 26.2 Chemoenzymatic commercial route to posaconazole key intermediate **8**.

1→3 acyl migration. This second-generation biocatalytic process [15,18] has been scaled up to provide multiple tons of the key (2*R*, 4*S*)-phenylsulfonate intermediate **8**.

The introduction of a new chemical route coupled with enzymatic desymmetrization of the diol resulted in the development of a highly efficient seven-step synthesis that utilizes inexpensive raw materials and features 35% overall yield with only one isolated intermediate (diol **12**). This represents a striking improvement when compared with the original 12-step approach using Sharpless epoxidation which provided only a 6% overall yield.

26.2.2 RIBAVARIN ALANINE ESTER

The supply route to a new antiviral candidate, ribavirin alanine ester (RAE), represents an example of establishing a biocatalytic route primarily for expedited deliveries of clinical supplies for phase I studies. This strategy provided an ample opportunity for developing a lower-cost chemical route to support large-scale deliveries of the drug substance.

TABLE 26.1
Comparison of the First- and Second-Generation Biocatalytic Routes to 13

	First Generation	Second Generation
Solvent	Acetonitrile	Acetonitrile
Diol concentration (g/L)	200	200
Enzyme loading (g/L)	10	10
Acylating agent	Vinyl acetate	Isobutyric anhydride
Temperature (°C)	0	–10 to –15
Reaction time (h)	6–8	6–8
Isolated yield (%)	74–81	90–95
ee (%)	97–99	98

Ribavirin is a potent antiviral agent used in combination with alfa-2 β interferon for the treatment of hepatitis C. Unfortunately, variations in the intersubject bioavailability of this drug have resulted in an increased risk of hemolysis and anemia in patients with high bioavailability, and low response in patients exhibiting low bioavailability. A series of preclinical evaluations indicated that ribavirin administered in the form of a prodrug, such as the alanine ester, afforded an improved pharmacokinetic profile. In order to provide support for toxicological and formulation development studies and early clinical trials, rapid access to 50 to 100 kg of the prodrug was required.

Synthesis of RAE centered on the formation of intermediate **19**, which was converted into the product **20** by hydrogenation in acetic acid (Figure 26.3). Attempts to obtain **19** by direct chemical acylation of the unprotected ribavirin with Cbz-Ala were not successful as they resulted in the formation of a mixture of mono-, di-, and triacylated products. A chemical strategy based on protection of the two secondary hydroxyls in the form of acetonide showed some promise. However, the projected 6-month timeline to scale up this three-step approach (Figure 26.4) would delay initiation of the clinical trial. To circumvent this problem, an alternative strategy based on a direct regioselective enzymatic acylation of ribavirin was proposed which obviated the need for protection/deprotection steps.

The use of CALB for the selective acylation of several 5'-hydroxy nucleosides with amino acid derivatives [19,20] prompted us to evaluate this enzyme for acylation of ribavirin with Cbz-Ala. The enzyme was found to be active and regioselective. Reaction conditions were optimized with respect to the solvent, temperature, and nature of the acylating agent used, enabling a fivefold improvement in yield when compared with initial conditions. Typically a yield of 85% of the 5'-acylated ribavirin was achieved in dry tetrahydrofuran (THF) at 60°C using the acetone oxime ester of Cbz-Ala as an acylating agent. As the coupling of **16** with acetone oxime proceeded in a nearly quantitative yield, we investigated

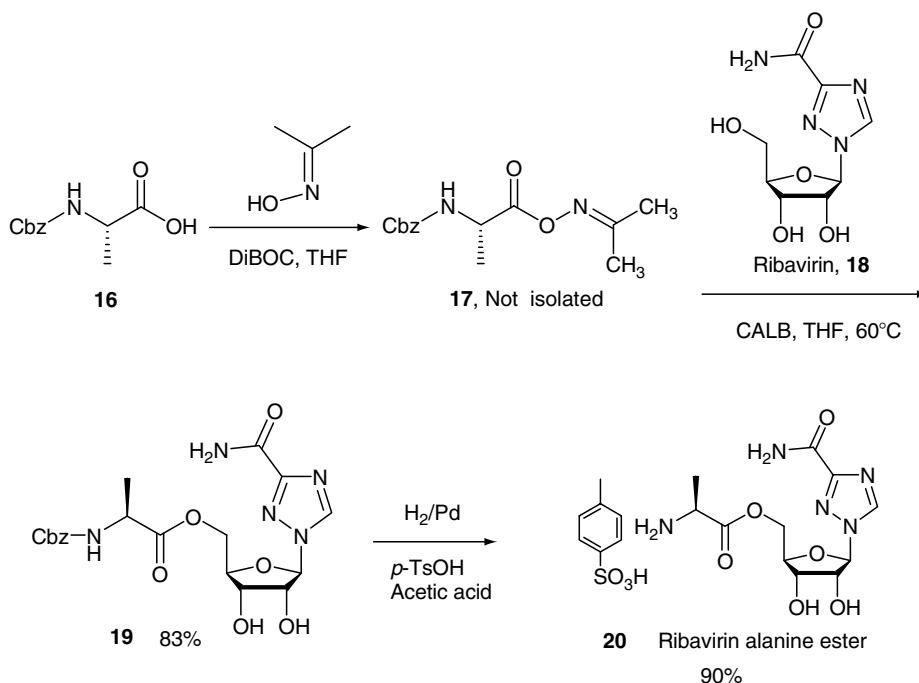


FIGURE 26.3 Chemoenzymatic supply route to ribavirin alanine ester (RAE) **20**.

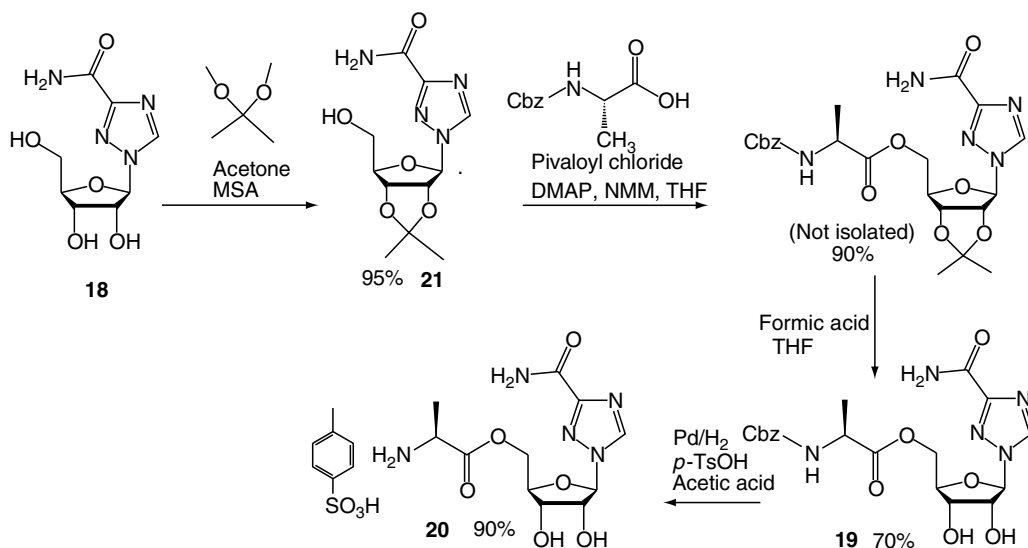


FIGURE 26.4 Chemical route to ribavirin alanine ester (RAE) **20**.

the option of running the acylation reaction without isolating intermediate **17** (Figure 26.3). To that end, **16** was esterified with acetone oxime, the reaction mixture was diluted threefold with THF, ribavirin was added, and acylation was initiated with the addition of the lipase. Minimizing moisture (<0.05%) in the solvent was found to be critical for achieving high product yield by minimizing lipase-catalyzed hydrolysis. The immobilized catalyst remained completely insoluble in THF at 60°C, forming a uniform free-flowing suspension. Even though most of the ribavirin also remained in a suspension (due to poor solubility in THF), the reaction rate was found to be independent of the rate of mixing, indicating that the rate of conversion was not controlled by diffusion. Following 24 h of incubation at 60°C, **19** was isolated in 85% yield, which was comparable to the yield obtained in the two-step procedure. The aforementioned process was transferred to the pilot plant where it was run under the conditions outlined in Table 26.2 to produce ~80 kg of **19** in 80 to 85% isolated yield and >98% purity.

The above supply strategy not only provided drug substance for formulation development and clinical trials, but also afforded ample opportunity to explore and develop an alternative chemical approach involving protection/deprotection of the two secondary

TABLE 26.2
Pilot Scale Reaction Conditions for Enzymatic
Acylation of Ribavirin **18**

Solvent	THF
Substrate loading	25 g/L
Temperature	55–60°C
Enzyme loading	20 g/L
Reaction time	24 h
Average yield	83%
Purity	99.3%
de	>99.9%

TABLE 26.3
Comparison of Chemical and Enzymatic Approaches to 20

Route	Chemical	Enzymatic
Steps	4	2
Isolated intermediates	2	1
Concentration of ribavirin (g/L)	100	25
Reagents cost/kg product (\$)	600–700	600–700
Enzyme cost/kg product (\$)	0	700

hydroxyls (Figure 26.4). Although both approaches were found to be commercially viable, the chemical route having a fourfold higher volumetric productivity proved to be more efficient and less costly (Table 26.3). Despite the fact that the biocatalytic approach was not utilized beyond the pilot scale, its rapid implementation accelerated the clinical program by about 6 months, providing a significant economic benefit to the company.

26.2.3 NK₁/NK₂ RECEPTOR ANTAGONIST

A new class of nonpeptide oxime-based antagonists of NK₁/NK₂ receptors have drawn much attention in recent years as potential drugs for treating a variety of chronic diseases including asthma, bronchospasm, arthritis, and migraine. The biological evaluation of one such antagonist, SCH 206272 (Figure 26.5), revealed that the affinity for the NK₂ receptor resided predominately in the *R,R*-diastereomer. The proposed approach to establishing the *R*-stereochemistry of the carbon adjacent to the oxime centered on desymmetrization of prochiral diethyl 3-[3',4'-dichlorophenyl] glutarate, **22**. The monoglutarate product, **23**, was then elaborated into the desired drug substance following the route illustrated in Figure 26.5. Unlike the two examples discussed earlier, in which the biological and the chemical approaches were investigated sequentially or in parallel, synthesis of SCH 206272 represents a paradigm in which the biological approach was the only one considered.

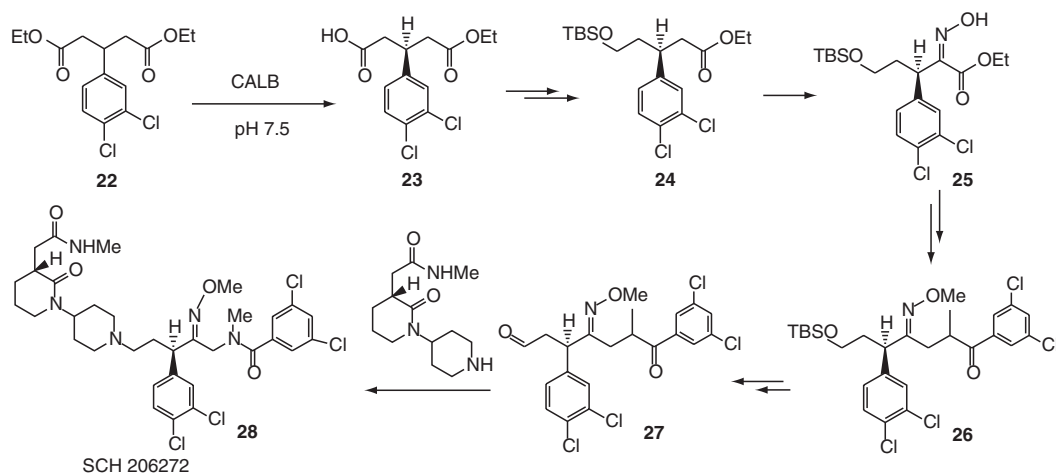


FIGURE 26.5 Chemoenzymatic supply route to NK₁/NK₂ receptor antagonist SCH 206272.

TABLE 26.4
Pilot-Scale Reaction Conditions for Selective Hydrolysis of **22**

Substrate loading	100 g/L
Immobilized enzyme (CALB)	12.5 g/L
pH	7.5
Temperature	38–40°C
Reaction time	18–24 h
Conversion yield	92–96%
ee	>99%
Enzyme cost/kg monoester	~\$200

Several enzymes capable of hydrolyzing **22** to both *S*- and *R*-**23** with good to excellent enantioselectivity were identified from a screen of ~200 commercial hydrolases [21]. Out of 11 candidates with *pro-S* selectivity, CALB was selected for further development due to the enzyme's excellent selectivity, moderate cost, and broad commercial availability. More than 95% conversion of 100 g/L of **22** to *S*-**23** was achieved in the presence of 20 g/L of immobilized CALB in 24 h at 40°C. Operating at temperatures above 37°C (the melting point of **22**) was critical for completing the conversion as the reaction rate increased sevenfold between 36 and 38°C, coinciding with the transition of **22** from the suspension to the emulsion state. No product inhibition was observed and the initial rate, as expected, was proportional to the concentration of the catalyst. Reducing the enzyme concentration by half resulted in a significant extension of time required to complete the reaction due to progressive inactivation of the catalyst. In fact, the immobilized lipase preparation lost ~30% of its original activity within the first 18 h. This result was somewhat unexpected in light of the commonly accepted view that CALB is a thermostable enzyme. The rate of inactivation was found to decrease at more neutral pH, which allowed us to reduce enzyme loading from 20 to 12.5 g/L. A 97% conversion of 100 g/L of **22** was achieved on a 50 to 70 kg scale in 18 to 24 h at 38 to 40°C as outlined in Table 26.4.

Given the low solubility of the substrate, the high reaction yield, and the excellent solubility of the product in aqueous medium at basic pH, product isolation was straightforward and easy to scale up. The enzyme preparation was not recycled due to partial inactivation of the catalyst.

Further investigations were conducted in pursuit of greater process efficiencies for commercial scale-up. The use of soluble enzyme as catalyst was examined to eliminate the need for the enzyme filtration step, thereby simplifying the purification procedure and possibly reducing the cost contribution of the immobilized enzyme. However, despite a slightly higher recovery yield of the isolated product, greater enzyme inactivation was observed with the soluble form of the enzyme. Consequently, the overall cost contribution of the soluble catalyst was about twice that of the immobilized enzyme preparation. In order to improve the process economics and reduce the catalyst cost, modification of CALB activity and/or stability through protein engineering was carried out.

26.3 DIRECTED EVOLUTION TO IMPROVE THE FUNCTIONAL PROPERTIES OF CALB

Various targeted protein-engineering techniques have been used to generate CALB variants with improved activity, thermostability, and enantioselectivity [22–24]. However, none of

these approaches has matched the success achieved by directed evolution technology [25–29]. For this reason, we attempted to improve two functional properties of CALB by utilizing the two most commonly used directed evolution strategies: error-prone polymerase chain reaction (PCR) for improving thermostability, and family shuffling for increasing specific activity for the selective hydrolysis of **22**.

26.3.1 APPLICATION OF ERROR-PRONE PCR FOR IMPROVING THERMOSTABILITY

Error-prone PCR is the most common approach to creating mutants of a single gene. This approach coupled with proper screening and/or selection often gives mutants with significantly altered functional characteristics [30–33]. To construct an error-prone PCR mutation library, we first cloned and expressed CALB in *Saccharomyces cerevisiae* to obtain secreted active N-terminal FLAG-fused protein. This strategy allowed for simple purification of recombinant CALB using anti-FLAG monoclonal antibodies. The cloned CALB gene was then subjected to error-prone PCR under conditions generating one to two amino acid changes in a protein molecule for each cycle of mutation, followed by screening for mutants with improved thermostability. Two high-throughput assays using commercially available 6,8-difluoro-4-methylumbelliferyl octanoate and *p*-nitrophenyl butyrate were developed and utilized for screening the library. The screening protocol involved incubating the mutant libraries for 1 h at 60 or 70°C and then determining their remaining activities at 20°C. Mutants having higher residual activity compared with wild-type CALB (WT-CALB) were selected for further characterization. This approach was expected to identify CALB variants with either higher melting point (T_m) or those with an increased propensity for refolding. Two rounds of error-prone PCR mutagenesis resulted in the isolation of two mutants, 23G5 and 195F1, having more than a 20-fold increase in half-life at 70°C but lower T_m compared with WT-CALB (Table 26.5). Circular dichroism (CD) and protein precipitation studies suggested that the increase in half-life of these two mutants was due to their diminished capacity to aggregate in the unfolded state and also to improved refolding efficiency. Sequence analysis revealed that the first-generation mutant, 23G5, had two amino acid mutations, V210I and A281E. The second-generation mutant, 195F1, derived from 23G5, had one additional mutation, V221D (Table 26.5). On the basis of structure modeling and CD analysis of the three individual variants at each mutation site, we concluded that A281E and V221D, but not V210I, were critical to improving the refolding efficiency of both mutants [34]. As expected, no improvement in activity was observed when these mutants were evaluated for hydrolysis of **22**.

26.3.2 APPLICATION OF DNA FAMILY SHUFFLING FOR IMPROVING ACTIVITY

Two homologous lipase B genes derived from *Hyphozyma* (designated P60) and *Cryptococcus* (designated P57) were cloned and expressed in *S. cerevisiae*. FLAG was fused to the N-termini of the enzymes to facilitate their secretion and to simplify isolation. The two aforementioned

TABLE 26.5
Amino Acid Substitutions, T_m , and $T_{1/2}$ of Wild-Type and Mutant CALB

Enzyme	Altered Amino Acid	T_m (°C)	$T_{1/2}$ (min)
WT-CALB	—	57.7	8
23G5	V210I, A281E	52.1	211
195F1	V210I, A281E, V221D	50.8	232

genes and the one coding for WT-CALB (designated P52) showed identities of 67 to 70% at the DNA level and 73 to 81% at the protein level. The three genes were used as parents to create a diverse library based on a DNA family shuffling procedure of *in vitro* and *in vivo* recombination of homologous genes in yeast [28]. Sequence and functional characterization of randomly picked clones revealed that the library was highly diverse at the molecular and functional levels. A pH indicator-based high-throughput screen was developed and used to identify chimeras having improved hydrolytic activity with **22**. Out of approximately 2500 screened variants, 69 demonstrated over a twofold improvement in the rate of hydrolysis when compared with the most active parent P57. Sixteen variants produced the desired (*S*)-monoglutarate **23** with >99% ee. Of these 16 clones, 7 had a 3- to 13-fold higher specific activity than P57, and up to a 20-fold higher activity than CALB (P52) as summarized in Table 26.6. Sequence analysis revealed that seven clones had no point mutations and contained DNA elements derived from two to three parents resulting from two to five crossover events [35].

CALB is a moderately thermostable enzyme, retaining activity for an extended period of time between 30 and 40°C. Above 40°C and especially in the presence of a high concentration of a water-insoluble substrate the enzyme becomes progressively unstable [21]. Since the P60 parent was reported to be more thermostable than CALB [36], we set out to investigate if the clones selected for increased activity toward the hydrolysis of **22** also acquired improved thermostability. Determination of T_m revealed that all seven variants had higher T_m values than that of CALB (Table 26.6). One of the two most thermostable variants, 3A4, exhibited a 11-fold longer half-life at 45°C and a 6.4°C higher T_m compared with CALB.

Having created several CALB variants with significantly improved thermostability and activity toward the hydrolysis of **22**, we then set out to develop a heterologous expression system to produce enzyme. Variant 3A4 was chosen for development for its combination of high specific activity (16-fold more than CALB) and thermostability (6.4°C more than CALB) [35].

TABLE 26.6
Specific Activity, $T_{1/2}$, and T_m of Parental and Chimeric Enzymes

Enzyme	Activity ^a	$T_{1/2}$ (h)	T_m (°C)
P52 (WT-CALB)	1.2	2	56.2
P57(<i>Cryptococcus</i>)	1.8	11	44 (62) ^c
P60 (<i>Hyphozyma</i>)	1.1	24	65.9
2A10	11	ND ^b	57.5
2B3	5.7	ND	59.5
3A4	20	24	62.6
4D6	24	ND	58.3
5D10	9.3	ND	62.6
8C7	16	ND	58.9
12E10	9.0	ND	61.6

^aAverage values (nmol/min/μg) were determined for the hydrolysis of **22** based on five assays having a standard deviation ≤10%.

^bNot determined.

^cBased on two separate transition phases in the unfolding profile.

26.4 PRODUCTION OF CHIMERIC LIPASE B BY RECOMBINANT FERMENTATION

Three microbial hosts were initially evaluated for CALB expression including *Aspergillus niger*, *S. cerevisiae*, and *Pichia pastoris*. Following the optimization of expression in flask fermentations, *P. pastoris* was selected for all further studies because it exhibited up to fourfold higher titers than the other two host systems (Table 26.7).

Numerous publications describe the use of *Pichia* systems to express proteins from a diverse array of sources [37–39]. Protein expression in *P. pastoris* is mediated by the highly inducible AOX1 promoter derived from the alcohol oxidase I gene. As recombinant expression is repressed by glycerol but induced by methanol, tight regulation of protein expression can be readily accomplished through manipulation of the carbon source. *Pichia* has a great propensity for respiratory growth, and therefore *Pichia*-based expression can be exploited to achieve high cell density affording high recombinant protein production.

As indicated in Section 26.3, chimera 3A4 expressed in *S. cerevisiae* exhibited the best improvement in thermostability and activity among the enzyme variants studied and was therefore chosen for pilot-scale development in 30 L fermentors. The 3A4 chimera gene was inserted into shuttle vector pPIC9K for expression in *P. pastoris*. Transformants selected on His[−] plates were screened for growth on medium plates containing the aminoglycoside G418. Prolonged exposure of transformants to increasing levels of G418 (0.25 to 4 mg/L) through sequential plate transfers employing selective and complex media enabled isolation of transformants with single and multicopy chromosomal inserts of the 3A4 chimera gene as confirmed by colony PCR. Interestingly, multicopy gene integration did not necessarily lead to higher enzyme production in complex media. Two transformants, one of the methanol-tolerant strain GS115 harboring a single gene copy, and the other of the methanol-sensitive strain KM71 with multiple gene copies, exhibited a similar level of hydrolytic activity. Both transformants provided a notable 50-fold improvement in expression compared with the original *S. cerevisiae*-derived 3A4. Further development of these transformants was pursued in minimal salts medium as recommended by Invitrogen.

TABLE 26.7
Secreted Expression Levels of CALB by Microbial Hosts

Host	Vector	Expression	Activity ^a
<i>Saccharomyces cerevisiae</i> (BJ505-protease deficient)	YEplFLAG-1 ADH2 Promoter TRP1 Selectable marker α -Factor secretion leader N-terminal FLAG tag	Mature sequence regulated by glucose repression	45 U
<i>Aspergillus niger</i> (ATCC 20739)	pBARGPE1 gpdA Promoter argB Selectable marker	Full-length sequence constitutive expression	39 U
<i>Pichia pastoris</i> (KM71)	pPIC9K AOX1 Promoter α -Factor secretion leader HIS4 Selectable marker	Mature sequence regulated by methanol induction	160 U

^aCALB Activity 1 Unit = 1 μ mol tributyrin hydrolyzed/min/mL supernatant.

In contrast to complex medium where both transformants provided similar levels of expression, in minimal salts medium the level of 3A4 expression in GS115 was reduced by a factor of 5. Consequently, the KM71 transformant was chosen for fermentation scale-up. Initial development was patterned after the approach developed by Jahic et al. [40], wherein recombinant CALB expression was regulated by either a methanol-limited or temperature-limited fed-batch fermentation strategy. The rationale for controlling expression by using growth-restricting temperatures was based on the observation that cellular lysis and concomitant extracellular proteolysis of CALB diminished at lower temperature. In this study, however, the difference in the degree of proteolysis occurring in the fermentation maintained at 28°C, and the one having a temperature transition from 28° to 10°C was negligible. Notably, decreasing the temperature from 28 to 20°C during glycerol feeding and maintaining the lower temperature throughout methanol feeding provided better control of DO ($\geq 5\%$ saturation), growth kinetics, and methanol concentration.

The growth rate in minimal salts medium was enhanced by initiating the fermentation at pH 6.3, allowing natural acidification to pH 5.0, and maintaining the pH by automatic addition of ammonium hydroxide (7N). Glycerol feeding (60% solution) administered for about 9 h at 2 to 3 mL/min followed by an overnight feed at half the rate allowed significant increases in cell density ($A_{600\text{ nm}} > 300$) while minimizing the level of residual glycerol prior to induction with methanol. Residual glycerol proved to be a mitigating factor in predisposing the culture to methanol toxicity. As methanol catabolism is repressed by glycerol, methanol feeding in the presence of glycerol was likely leading to transient elevation of methanol to toxic levels. Hence, methanol feed rates were adjusted based on frequently performed enzymatic analysis of glycerol and methanol content using an oxygen probe-based analyzer (Analox GM8, Lunenburg, Massachusetts). In the course of fermentation, the culture was progressively adapted to methanol catabolism by feeding a 25% methanol solution (at 0.5 to 1.0 mL/min) for the first 7 h, followed by a 50% methanol solution for the next 18 h, and finally feeding 100% methanol for the duration of the fermentation. Methanol feed rate, agitation, and temperature were adjusted to support cell growth and enzyme expression while maintaining a nontoxic methanol concentration ($< 0.5\%$). Particular attention was also given to controlling foam formation throughout growth. As depicted in [Figure 26.6](#), with proper control of methanol feeding, methanol concentrations were maintained below the toxic level ($< 0.5\%$ v/v), resulting in high culture densities ($A_{600\text{ nm}} \geq 500$) with chimera 3A4 lipase production levels of 1.2 to 1.5 g/L following 10 d of fermentation.

Secreted enzyme was isolated from the fermentation by first removing cells by centrifugation ($10,000 \times g$, 15 min), followed by concentration of the remaining supernatant ~ 15 -fold by ultrafiltration using 10 kD exclusion membrane and lyophilization. The lyophilized material was purified by passing the crude suspension over a mono-Q anion exchange column that retained most of the contaminating proteins. Over 10 g of purified chimeric 3A4 lipase was obtained using 20 L of clarified supernatant. When compared with purified wild-type CALB expressed in *P. pastoris* (KM71), chimera 3A4 lipase exhibited a tenfold higher specific activity (8.6 nmol/min/ μg protein) in the selective desymmetrization of **22**.

26.5 CONCLUSIONS

Today biocatalysis serves a multitude of functions ranging from discovery-type synthesis of leading drug candidates and elaboration of bioactive compound libraries, to generation of drug metabolites. As revealed by the case studies presented in this chapter, biocatalysis has been used successfully as a means to expedite drug substance supplies for toxicological and clinical studies, as a primary approach to key drug intermediates, and as enabling technology

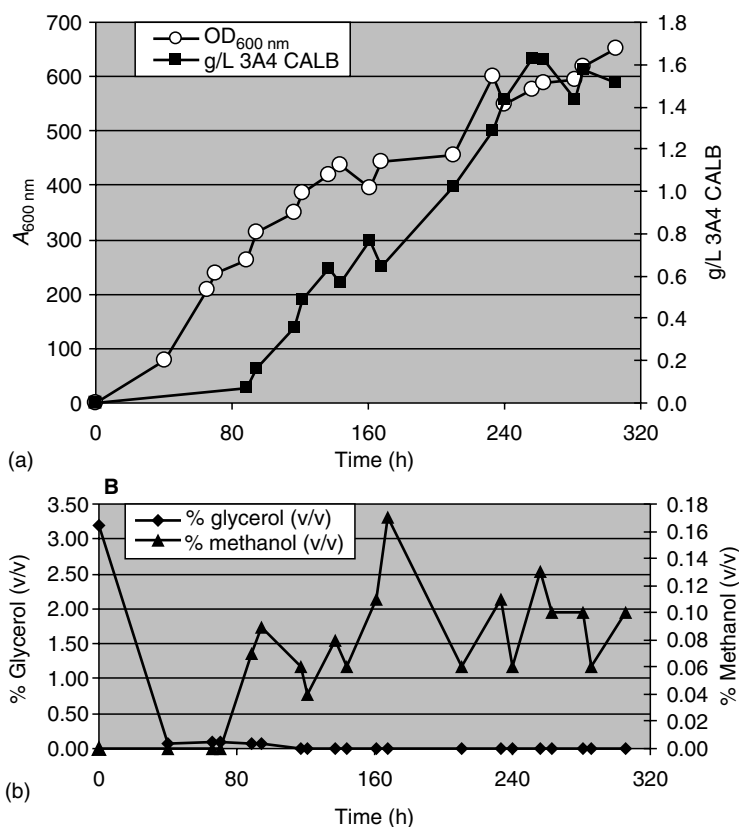


FIGURE 26.6 Expression of CALB 3A4 chimera in *Pichia pastoris* in 30 L fermentors. (a) Cell density and CALB expression level. (b) Residual glycerol and methanol concentrations.

in commercial production displacing initial chemical approaches. By incorporating protein engineering and recombinant expression technologies, biocatalysts can now be tailored to meet continuously evolving process criteria. As such, future developments will likely reveal greater reliance on biocatalysts to provide the “best” process route for production of pharmaceuticals.

ACKNOWLEDGMENTS

The authors would like to thank G. Wong, W. Tong, R. Raghavan, and J. Park for their contribution to the chemical synthesis of ribavirin alanine ester, as well as all the chemists, engineers, and operators in Chemical Development who supported process development of the three developmental candidates described in this chapter.

REFERENCES

1. Breuer, M., Ditrach, K., Habicher, T., Hauer, B., Kessler, M., Sturmer, R., and Zelinski, T., Industrial methods for the production of optically active intermediates, *Angew. Chem. Int. Ed. Eng.*, 43(7), 788–824, 2004.
2. Panke, S. and Wubbolts, M., Advances in biocatalytic synthesis of pharmaceutical intermediates, *Curr. Opin. Chem. Biol.*, 9(2), 188–194, 2005.

3. Michels, P.C., Khmelnitsky, Y.L., Dordick, J.S., and Clark, D.S., Combinatorial biocatalysis: a natural approach to drug discovery, *Trends Biotechnol.*, 16(5), 210–215, 1998.
4. Krstenansky, J.L. and Khmelnitsky, Y., Biocatalytic combinatorial synthesis, *Bioorg. Med. Chem.*, 7(10), 2157–2162, 1999.
5. Rich, J.O., Michels, P.C., and Khmelnitsky, Y.L., Combinatorial biocatalysis, *Curr. Opin. Chem. Biol.*, 6(2), 161–167, 2002.
6. Secundo, F., Carrea, G., De Amici, M., Joppolo Di Ventimiglia, S., and Dordick, J.S., A combinatorial biocatalysis approach to an array of cholic acid derivatives, *Biotechnol. Bioeng.*, 81(4), 391–396, 2003.
7. Reiss, P., Burnett, D.A., and Zaks, A., An enzymatic synthesis of glucuronides of azetidinone-based cholesterol absorption inhibitors, *Bioorg. Med. Chem.*, 7(10), 2199–2222, 1999.
8. Kamimori, H., Ozaki, Y., Okabayashi, Y., Ueno, K., and Narita, S., Synthesis of acylglucuronides of drugs using immobilized dog liver microsomes octadecylsilica particles coated with phospholipid, *Anal. Biochem.*, 317(1), 99–106, 2003.
9. Linnet, K., Glucuronidation of olanzapine by cDNA-expressed human UDP-glucuronosyltransferases and human liver microsomes, *Hum. Psychopharmacol.*, 17(5), 233–238, 2002.
10. Kuuranne, T., Aitio, O., Vahermo, M., Elovaara, E., and Kostianen, R., Enzyme-assisted synthesis and structure characterization of glucuronide conjugates of methyltestosterone (17 α -methylandrosta-4-en-17 β -ol-3-one) and nandrolone (estr-4-en-17 β -ol-3-one) metabolites, *Bioconjug. Chem.*, 13(2), 194–199, 2002.
11. Kren, V., Ulrichova, J., Kosina, P., Stevenson, D., Sedmera, P., Prikrylova, V., Halada, P., and Simanek, V., Chemoenzymatic preparation of silybin β -glucuronides and their biological evaluation, *Drug Metab. Dispos.*, 28(12), 1513–1517, 2000.
12. Vail, R.B., Homann, M.J., Hanna, I., and Zaks, A., Preparative synthesis of drug metabolites using human cytochrome P450s 3A4, 2C9 and 1A2 with NADPH-P450 reductase expressed in *Escherichia coli*, *J. Ind. Microbiol. Biotechnol.*, 32(2), 67–74, 2005.
13. Saksena, A.K., Girijavallabhan, V.M., Pike, R.E., Wang, H., Lovey, R.G., Liu, Y.-T., Ganguly, A.K., Morgan, W.B., and Zaks, A., Preparation of chiral 2-azolylmethyl-2-phenyl-4-sulfonyloxymethyltetrahydrofurans as antifungal intermediates, WO 9425452, 1994.
14. Lovey, R.G., Saksena, A.K., and Girijavallabhan, V.M., PPL-catalyzed enzymic asymmetrization of a 2-substituted prochiral 1,3-diol with remote chiral functionality: improvements toward synthesis of the eutomers of SCH 45012, *Tetrahedron Lett.*, 35(33), 6047–6050, 1994.
15. Morgan, B., Dodds, D.R., Homann, M.J., Zaks, A., and Vail, R., Biocatalysis in pharmaceutical process development: SCH56592, a case study, *Methods Biotechnol.*, 15(Enzymes in Nonaqueous Solvents), 423–467, 2001.
16. Morgan, B., Dodds, D.R., Zaks, A., Andrews, D.R., and Klesse, R., Enzymic desymmetrization of prochiral 2-substituted-1,3-propanediols: a practical chemoenzymic synthesis of a key precursor of SCH 51048, a broad-spectrum orally active antifungal agent, *J. Org. Chem.*, 62(22), 7736–7743, 1997.
17. Martinelle, M. and Hult, K., Kinetics of acyl transfer reactions in organic media catalyzed by *Candida antarctica* lipase B, *Biochim. Biophys. Acta*, 1251(2), 191–197, 1995.
18. Nielsen, C.M. and Sudhakar, A., Process for preparing intermediates for the synthesis of antifungal agents, WO 9722710, 1997.
19. Moris, F. and Gotor, V., Selective aminoacylation of nucleosides through an enzymatic reaction with oxime aminoacyl esters, *Tetrahedron*, 50(23), 6927–6934, 1994.
20. Mahmoudian, M., Eaddy, J., and Dawson, M., Enzymatic acylation of 506U78 (2-amino-9- β -D-arabinofuranosyl-6-methoxy-9H-purine), a powerful new anti-leukemic agent, *Biotechnol. Appl. Biochem.*, 29(3), 229–233, 1999.
21. Homann, M.J., Vail, R., Morgan, B., Sabesan, V., Levy, C., Dodds, D.R., and Zaks, A., Enzymatic hydrolysis of a prochiral 3-substituted glutarate ester, an intermediate in the synthesis of an NK1/NK2 dual antagonist, *Adv. Synth. Catal.*, 343(6&7), 744–749, 2001.
22. Patkar, S.A., Svendsen, A., Kirk, O., Clausen, I.G., and Borch, K., Effect of mutation in non-consensus sequence Thr-X-Ser-Gly of *Candida antarctica* lipase B on lipase specificity, specific activity and thermostability, *J. Mol. Catal. B Enzym.*, 3, 51–54, 1997.
23. Patkar, S., Vind, J., Kelstrup, E., Christensen, M.W., Svendsen, A., Borch, K., and Kirk, O., Effect of mutations in *Candida antarctica* B lipase, *Chem. Phys. Lipids*, 93, 95–101, 1998.

24. Rotticci, D., Rotticci-Mulder, J.C., Denman, S., Norin, T., and Hult, K., Improved enantioselectivity of a lipase by rational protein engineering, *Chem. Biochem.*, 2, 766–770, 2001.
25. May, O., Nguyen, P.T., and Arnold, F.H., Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine, *Nat. Biotechnol.*, 18, 317–320, 2000.
26. Ness, J.E., Welch, M., Giver, L., Bueno, M., Cherry, J.R., Borchert, T.V., Stemmer, W.P. C., and Minshull, J., DNA shuffling of subgenomic sequences of subtilisin, *Nat. Biotechnol.*, 17, 893–896, 1999.
27. Ness, J.E., Kim, S., Gottman, A., Pak, R., Krebber, A., Borchert, T.V., Govindarajan, S., Mundorff, E.C., and Minshull, J., Synthetic shuffling expands functional protein diversity by allowing amino acids to recombine independently, *Nat. Biotechnol.*, 20, 1251–1255, 2002.
28. Abecassis, V., Pompon, D., and Truan, G., High efficiency family shuffling based on multi-step PCR and *in vivo* DNA recombination in yeast: statistical and functional analysis of a combinatorial library between human cytochrome p450 1A1 and 1A2, *Nucleic Acids Res.*, 28, 1–10, 2000.
29. Christians, F.C., Scapozza, L., Cramer, A., Folkers, G., and Stemmer, W.P.C., Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling, *Nat. Biotechnol.*, 17, 259–264, 1999.
30. Chen, Z. and Zhao, H., Rapid creation of a novel protein function by *in vitro* coevolution, *J. Mol. Biol.*, 348(5), 1273–1282, 2005.
31. Henke, E. and Bornscheuer, U.T., Directed evolution of an esterase from *Pseudomonas fluorescens*: random mutagenesis by error-prone PCR or a mutator strain and identification of mutants showing enhanced enantioselectivity by a resorufin-based fluorescence assay, *Biol. Chem.*, 380, 1029–1033, 1999.
32. Song, J.K. and Rhee, J.S., Enhancement of stability and activity of phospholipase A₁ in organic solvents by directed evolution, *Biochim. Biophys. Acta*, 1547, 370–378, 2001.
33. Zha, D., Wilense, S., Hermes, M., K.-E. Jaeger, and Reetz, M.T., Complete reversal of enantioselectivity of an enzyme-catalyzed reaction by directed evolution, *Chem. Commun.*, 2664–2665, 2001.
34. Zhang, N., Suen, W.-C., Windsor, W., Xiao, L., Madison, V., and Zaks, A., Improving tolerance of *Candida antarctica* lipase B towards irreversible thermal inactivation through directed evolution, *Protein Eng.*, 16(8), 599–605, 2003.
35. Suen, W.-C., Zhang, N., Xiao, L., Madison, V., and Zaks, A., Improved activity and thermostability of *Candida antarctica* lipase B by DNA family shuffling, *Protein Eng. Des. Sel.*, 17(2), 133–140, 2004.
36. Hashida, M., Abo, M., Takamura, Y., Kirk, O., Halkier, T., Pedersen, S., Patkar, S.A., and Hansen, M.T., U.S. Patent 5,856,163, 1999, Lipases from *Hyphozyma*.
37. Cregg, J., Cereghino, J., Shi, J., and Higgins, D., Recombinant protein expression in *Pichia pastoris*, *Mol. Biotechnol.*, 16(1), 23–52, 2000.
38. Zhang, W., Inan, M., and Meagher, M., Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*, *Biotechnol. Bioprocess Eng.*, 5(4), 275–287, 2000.
39. Lin Cereghino, G., Sunga, A., Lin Cereghino, J., and Cregg, J., Expression of foreign genes in the yeast *Pichia pastoris*, *Genet. Eng. (New York)*, 23, 157–169, 2001.
40. Jahic, M., Wallberg, F., Bollok, M., Garcia, P., and Enfors, S.-O., Temperature limited fed-batch techniques for control of proteolysis in *Pichia pastoris* bioreactor cultures, *Microb. Cell Factories*, 2(6), 1–11, 2003.

27 Industrial Processes Using Lyases for C–C, C–N, and C–O Bond Formation

Martina Pohl and Andreas Liese

CONTENTS

27.1	Introduction	661
27.2	C–C Bond Formation	662
27.2.1	Pyruvate Decarboxylase	662
27.2.2	Deoxyribose-5-Phosphate Aldolase	663
27.2.3	<i>N</i> -Acetyl-D-Neuraminic Acid Aldolase	663
27.2.4	Tryptophan Synthase	664
27.2.5	Hydroxynitrile Lyase	665
27.3	C–N Bond Formation	665
27.3.1	Tyrosinphenol Lyase	665
27.3.2	Aspartase	667
27.3.3	Aspartate-4-Decarboxylase	668
27.3.4	L-Phenylalanine Ammonia Lyase	668
27.4	C–O Bond Formation	669
27.4.1	Fumarase	669
27.4.2	Malease	669
27.4.3	Nitrile Hydratase	670
27.4.4	Carnitine Dehydratase	671
27.5	Conclusion	672
	References	672

27.1 INTRODUCTION

Industrial biocatalysis is becoming a standard tool in the synthesis of fine chemicals [1,2]. Although a major number of industrially applied biotransformations is still catalyzed by hydrolases (E.C. 3.x.x.x) [3], oxidoreductases (E.C. 1.x.x.x), and lyases (E.C. 4.x.x.x), become more and more important. This chapter gives an overview of the application of lyases in industrial processes, either as isolated enzymes or as immobilized enzymes, and by whole cell biocatalysis. In contrast to hydrolases, which in general catalyze kinetic resolutions, lyases are usually employed for asymmetric syntheses, enabling 100% conversion and 100% (enantio) selectivity under ideal conditions [4].

27.2 C–C BOND FORMATION

The first asymmetric C–C bond formation catalyzed by hydroxynitrile lyase, from *Prunus amygdalins*, was described in 1909 using bitter almond meal [5]. In addition to hydroxynitrile lyases, aldolases, ketolases, and a certain type of thiamine-diphosphate dependent lyases play important roles in industrial biotransformations.

27.2.1 PYRUVATE DECARBOXYLASE

Pyruvate decarboxylases (E.C. 4.1.1.1) (synonym: PDC; systematic name: 2-oxo-acid carboxy-lyase) are tetrameric enzymes requiring thiamine diphosphate and magnesium ions as cofactors. They are found in many types of yeast, fungi, plants, and some bacteria. Apart from their physiological role, the decarboxylation of pyruvate to acetaldehyde, these enzymes have the potential to ligate two aldehyde molecules enantioselectively to 2-hydroxy ketones. The carboligation of acetaldehyde **1** and benzaldehyde **2** to (*R*)-phenylacetylcarbinol **3** (PAC) was one of the first biotransformations applied in industrial scale for the production of intermediates in the synthesis of fine chemicals (Figure 27.1).

PAC is not a marketed product, but is produced as a chiral precursor for various drugs having α - and β -adrenergic properties, such as (1*R*,2*S*)-ephedrine, (1*R*,2*R*)-pseudoephedrine, norephedrine, and norpseudoephedrine. Although (1*R*,2*S*)-ephedrine is a natural product found in various plant species of genus *Ephedra*, extraction from the natural source is not competitive to its biocatalytic or chemical synthesis. The fermentative process is still performed, by fermenting *Saccharomyces cerevisiae* cells in a fed-batch reactor, by BASF AG (Germany) and Krebs Biochemicals Ltd./Malladi Drug (India). Typical concentrations of (*R*)-PAC produced during yeast fermentation are 4 to 12 g/L. The flood of recent publications shows that process and strain optimization is still a matter of strong interest [6–9]. Most of the parameters that have been investigated so far are summarized [10,11]. Product concentrations were improved up to 12 to 20 g/L (*R*)-PAC by optimization of the fermentative procedure and the benzaldehyde feeding process [12]. Apart from *S. cerevisiae*, PDC from *Candida utilis* and *Rhizopus javanicus* have proved their potential for the production of PAC [12,13]. In addition to optimization of the whole-cell biotransformation, recent work focused on the development of processes with isolated PDC to circumvent problems of side product formation caused by various enzymes in whole-cell systems. In such enzymatic biotransformations product concentrations up to 100 g/L PAC were obtained [7,12].

A similar carboligase reaction has also been described by other thiamine-diphosphate dependent enzymes like benzoylformate decarboxylase (BFD), phenylpyruvate decarboxylase, and benzaldehyde lyase. Although these enzymes are not yet commercially applied, they have the potential for the enantioselective synthesis of a broad range of 2-hydroxy ketones as synthons for organic chemistry [14–16]. The differences and similarities between PDC and BFD were recently demonstrated by site-directed mutagenesis [17].

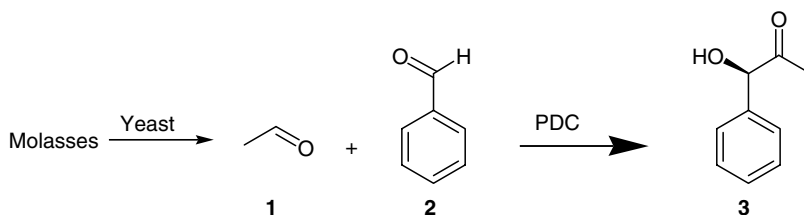


FIGURE 27.1 PDC catalyzed synthesis of (*R*)-phenylacetylcarbinol (PAC) **3**.

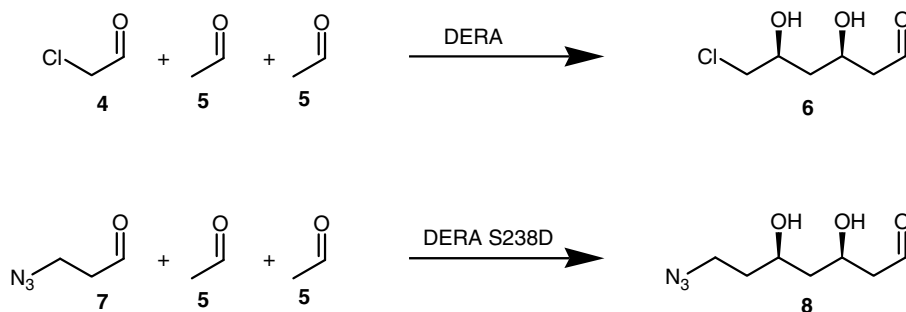


FIGURE 27.2 Deoxyribose-5-phosphate aldolase (DERA) as biocatalyst in the synthesis of intermediates **6** and **8** for (HMG-CoA) reductase inhibitors (statins).

27.2.2 DEOXYRIBOSE-5-PHOSPHATE ALDOLASE

Deoxyribose-5-phosphate aldolase (E.C. 4.1.2.4) (synonyms: DERA, phosphodeoxyriboaldolase, deoxyriboaldolase, deoxyribose-5-phosphate aldolase, 2-deoxyribose-5-phosphate aldolase; systematic name: 2-deoxy-D-ribose-5-phosphate acetaldehyde-lyase).

Wong et al. first described the potential of DERA to catalyze the aldol condensation of chloroacetaldehyde **4** with two molecules of acetaldehyde **5** yielding (3*R*,5*S*)-6-chloro-3,5-dihydroxyhexanal **6** (Figure 27.2). This chiral compound is an important precursor in the synthesis of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors (statins), hypolipidaemic agents, which are multibillion-dollar drugs [4]. The need for high enantioselectivity at both stereo centers has led to the development of six different routes involving biocatalysis [18,19]. The product **6** (97% de) of the DERA biotransformation is stabilized as hemiacetal under optimized process conditions at DSM. Independently, a similar approach utilizing DERA aldolase was also developed by the Wong group together with DIVERSA Coop. (USA) [20]. To broaden the range of accepted substrates they isolated a DERA variant (S238A), which accepts azidopropionaldehyde **7** alternatively to chloroacetaldehyde enabling access to 7-azido-(3*R*,5*S*)-dihydroxy-heptanal **8**, the key intermediate for Atorvastatin [21] (Figure 27.2).

27.2.3 *N*-ACETYL-D-NEURAMINIC ACID ALDOLASE

N-Acetyl-D-neuraminic acid aldolases (E.C. 4.1.3.3) (synonyms: Neu5Ac-aldolase, *N*-acetylneuraminate lyase, *N*-acetylneuraminate pyruvate-lyase, sialic acid aldolase; systematic name: *N*-acetylneuraminate pyruvate-lyase) have been characterized from many organisms. Many genes are cloned and three-dimensional (3D) structures of the enzymes from *Escherichia coli* and *Haemophilus influenzae* are available [22–23]. *In vivo* Neu5Ac-aldolase catalyzes the reversible aldol reaction of *N*-acetyl-D-mannosamine (ManNAc) **10** and pyruvate **11** to *N*-acetyl-5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid (Neu5Ac) **12**. Neuraminic acid, a C-9 amino sugar, is the aldol condensation product of pyruvic acid and *N*-acetyl-D-mannosamine, mostly important as its nitrogen- and oxygen-substituted *N*-acyl derivatives (sialic acids) that are found as the terminal sugar of cell surface glycoproteins, especially in animal tissue and blood cells. Sialic acids are components of lipids, polysaccharides, and mucoproteins, as cell surface glycoproteins that play an important role in cell adhesion, recognition, and interaction. The Neu5Ac analogs and Neu5Ac-containing oligosaccharides are precursors for inhibitors of neuraminidase, hemagglutinin, and selectin-mediated leucocyte adhesion [24]. For industrial biotransformation, the enzyme from

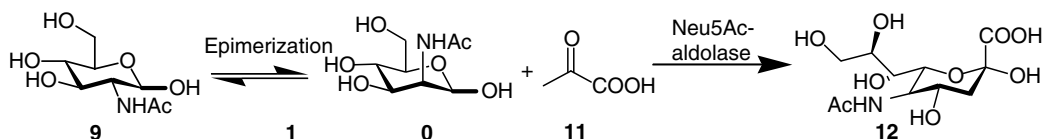


FIGURE 27.3 Synthesis of Neu5Ac **12** in a reaction sequence starting from *N*-acetyl-D-glucosamine **9** by epimerization at C2.

E. coli is used either immobilized on Eupergit-C (GlaxoSmithKline, UK) or in its solubilized form (Marukin Shoyu Co. Ltd., Japan, Research Center Jülich, Germany).

As *N*-acetyl-D-mannosamine **10** is very expensive, it is synthesized from *N*-acetyl-D-glucosamine **9** by epimerization at C2. The equilibrium of the epimerization is on the side of *N*-acetyl-D-glucosamine (GlcNAc:ManNAc = 4:1). After neutralization and addition of isopropanol, GlcNAc precipitates leaving behind a ratio of GlcNAc:ManNAc = 1:4 in the remaining solution. This chemical epimerization is used in the multiton production process of Neu5Ac in a repetitive batch process by GlaxoSmithKline [25,26]. By contrast, Marukin Shoyu Co. Ltd. and the Research Center Juelich integrated enzymatic epimerization using *N*-acetyl-D-glucosamine-2-epimerase from *E. coli* in a one-pot biotransformation (Figure 27.3). Downstream processing of Neu5Ac can easily be achieved by crystallization upon acidification with acetic acid [27,28]. The fed-batch process of Marukin Shoyu Co. Ltd., which is operated in a multikilogram scale, requires an additional thermal denaturation step (80°C, 5 min) to remove the enzymes from the product.

27.2.4 TRYPTOPHAN SYNTHASE

Tryptophan synthase (E.C. 4.2.1.20) (synonyms: tryptophan desmolase, L-tryptophan synthetase, indoleglycerol phosphate aldolase, systematic name: L-serine hydro-lyase) is a dimeric pyridoxal-5'-phosphate-dependent enzyme, which has been found in a range of organisms [22]. The alpha-subunit catalyzes the conversion of 1-(indol-3-yl)glycerol-3-phosphate to indole and glyceraldehydes-3-phosphate. The indole then migrates to the beta-subunit where, with serine in the presence of pyridoxal-5'-phosphate, it is converted to tryptophane [29]. For commercial purposes, the ability of the enzyme to catalyze the conversion of serine **13** and indole **14** to tryptophan **15** and water is used for the production of L-tryptophan as a pharmaceutical active ingredient for parenteral nutrition (infusion solution) as well as active ingredient in sedativa, neuroleptica, antidepressiva, and food additives. In the commercial process run at the Amino GmbH (Germany), the enzyme from *E. coli* is used in the form of suspended whole cells (Figure 27.4) [30]. The substrate L-serine is separated from molasse by ion exchange chromatography. The fed-batch is pH

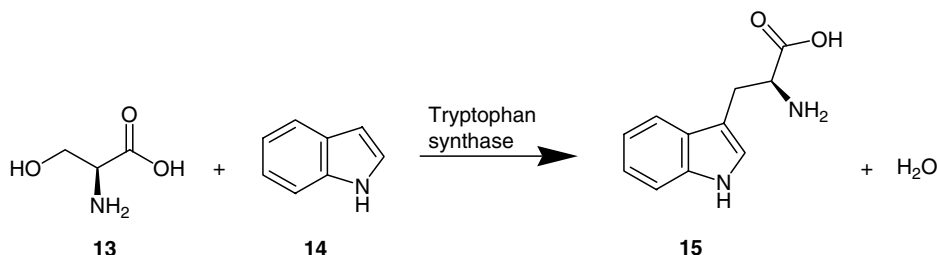


FIGURE 27.4 Synthesis of L-tryptophan **15** starting from L-serine **13** and indole **14**.

regulated and the indole dosage is controlled through online HPLC analysis of the product/educt ratio. Based on indole >95% yield is obtained in a fed-batch reactor. The process is run with 30 t/y.

27.2.5 HYDROXYNITRILE LYASE

Hydroxynitrile lyases (E.C. 4.1.2.X) (synonyms: HNL, oxynitrilase, mandelonitrile lyase, (*R*)-hydroxynitrile lyase, (*S*)-hydroxynitrile lyase, hydroxymandelonitrile lyase; systematic names: mandelonitrile benzaldehyde-lyase (E.C. 4.1.2.10), (*S*)-4-hydroxymandelonitrile hydroxybenzaldehyde-lyase (E.C. 4.1.2.11), acetone-cyanohydrin acetone-lyase (E.C. 4.1.2.39)) are enzymes that are originated in plants. This class of enzymes is a good example for convergent evolution, because hydroxynitrile lyase activity was introduced in different frameworks of oxidoreductases and α/β -hydrolases yielding hydroxynitrile lyases differing in enantioselectivity and substrate range [31]. A detailed study of 3D structures and mutant enzymes led to good understanding of the reaction mechanisms of (*R*)-specific mandelonitrile lyase from *Prunus* sp. (E.C. 4.1.2.10), acetone-cyanohydrin lyase from *Linum usitatissimum* (E.C. 4.1.2.37), (*S*)-specific hydroxymandelonitrile lyase from *Sorghum bicolor* (E.C. 4.1.2.11), and (*S*)-hydroxynitrile lyases (acetone-cyanohydrin acetone-lyases, E.C. 4.1.2.39) from *Hevea brasiliensis* and from *Manihot esculenta* [32]. Their ability to catalyze the cleavage of HCN from natural cyanide compounds, which is probably part of a plant defence system against herbivores and microbial attack, is synthetically applied for the synthesis of chiral hydroxynitriles [33–38]. The (*R*)-specific HNL from almond (*P. amygdalus*, *PaHNL*) [39] and two (*S*)-specific HNLs from rubber tree (*H. brasiliensis*, *HbHNL*), and cassava (*M. esculenta*, *MeHNL*) are nowadays available by overexpression in *Pichia pastoris*, *S. cerevisiae*, and *E. coli*, respectively [40–42]. This paved the way for the introduction of HNL-based industrial processes for the production of chiral hydroxynitriles and 2-hydroxycarboxylic acid [2,31].

PaHNL has recently been implemented in industrial syntheses of some chiral aromatic 2-hydroxycarboxylic acids, such as (*R*)-2-chloromandelic acid **18**, an intermediate for the synthesis of the antidepressant and platelet-aggregation inhibitor clopidogrel by DSM Fine Chemicals Austria, Nippon Shokubai, and Clariant (Figure 27.5) [2,43–46]. For the enantioselective addition of HCN **17** to 2-chlorobenzaldehyde **16**, *PaHNL* is applied in the form of almond-flour extract or is immobilized on Avicel microcrystalline cellulose. Depending on the solvent employed the enzyme can be used for several months. The (*R*)-2-chloromandelonitrile formed is converted into the corresponding carboxylic acid by hydrolysis with concentrated HCl, without racemization. Thus, 100% theoretical yield is possible. One recent example is the production of (*S*)-3-phenoxybenzaldehyde cyanohydrin **20** in a biphasic process [47] by DSM Fine Chemicals Austria and Nippon Shokubai [47–50]. The cyanohydrin is a chiral intermediate in the production of pyrethroids [2]. Recently, the carving of the active site of almond *R*-HNL by means of site-directed mutations for increased enantioselectivity was demonstrated in respect to (*R*)-2-hydroxy-4-phenylbutyronitrile **22**, the key intermediate in the synthesis of different angiotensin-converting enzyme inhibitors [51].

27.3 C–N BOND FORMATION

27.3.1 TYROSINPHENOL LYASE

Tyrosinphenol lyase (E.C. 4.1.99.2) (synonyms: beta-tyrosinase, TLP; systematic name: L-tyrosine phenol-lyase (deaminating)) is a pyridoxal-5'-dependent multifunctional enzyme that catalyzes degradation of tyrosine to phenol, pyruvate, and ammonia. The reversibility of

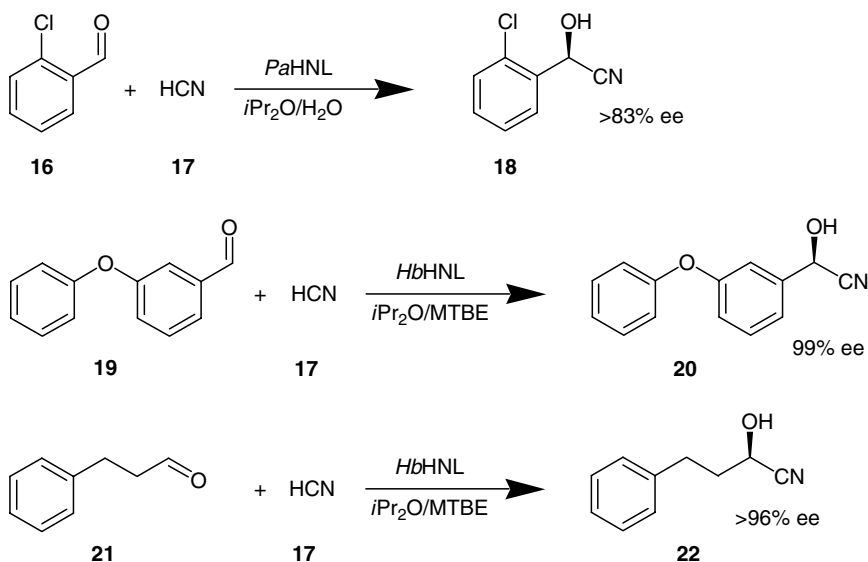


FIGURE 27.5 Asymmetric HCN addition to aldehydes catalyzed by hydroxynitrile lyases. *PaHNL*, hydroxynitrile lyase from *Prunus amygdalus* and *HbHNL*, hydroxynitrile lyase from *Manihot esculenta*.

this reaction is used for the production of L-DOPA using catechol instead of phenol as a substrate (Figure 27.6).

From a thorough strain screening, bacterium *Erwinia herbicola* was selected for L-DOPA production and the commercially applied process using suspended whole cells was optimized intensively [52]. Ajinomoto Co. Ltd. (Japan) produces L-DOPA **26** by this lyase-biotransformation using suspended *E. herbicola* cells with extremely high TLP activity in a fed-batch reactor [53]. First, cells are prepared by cultivation in a medium containing L-tyrosine as an inducer of TLP. The intact cells are then harvested by centrifugation and transferred to the reactor together with the substrate catechol **23**. The one-step biotransformation is more economic than the established chemical route that involves eight chemical reaction steps including optical resolution [52,54]. L-DOPA **26** is insoluble in the reaction medium, so it precipitates during the reaction from a final concentration of 110 g/L [52]. About 250 t of L-DOPA are supplied per year, and more than half of it are produced through biotransformation.

The product is applied for the treatment of Parkinsonism that is caused by a lack of L-dopamine and its receptors in the brain. *In vivo* L-dopamine is synthesized by decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA). Since L-dopamine cannot pass the blood–brain barrier L-DOPA is applied in combination with dopadecarboxylase-inhibitors to avoid formation of L-dopamine outside the brain.

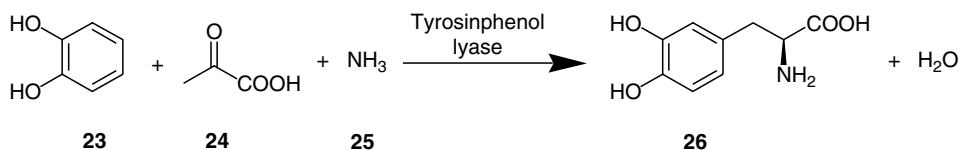


FIGURE 27.6 Tyrosinphenol lyase catalyzed L-DOPA **26** synthesis.

27.3.2 ASPARTASE

Aspartase (E.C. 4.3.1.1) (synonyms: L-aspartase, aspartate ammonia lyase, fumaric aminase; systematic name: L-aspartate ammonia lyase) enzyme was identified and cloned from various bacteria and the 3D structure from the tetrameric enzyme from *E. coli* was solved [22]. Aspartase does not require cofactors to catalyze the reversible cleavage of ammonia **28** from aspartate **29** to fumarate **27**. The reverse reaction is used for the production of L-aspartate **29**, since 1953 (Figure 27.7) [55], which is used in parenteral nutrition, as food additives and as a starting material for the low-calorie sweetener aspartame. Different procedures using whole cells and isolated aspartase, either mobilized or immobilized, have been developed for already four decades.

The industrial L-aspartate production by biotransformation was started using *E. coli* cells with high enzyme activity in a batch fermentation of fumaric acid and ammonia. This procedure had the disadvantage that cells with active enzyme had to be discarded at the end of each batch [55]. On the one hand, to overcome this problem, Tanabe Seiyaku Co. (Japan) produced L-aspartate, since 1973, using immobilized aspartase that was isolated from *E. coli* cells. The enzyme was bound to a weak ion exchange resin and since 1974, this procedure was used for L-aspartate production at Kyowa Hakko Kogyo Co. Ltd. On the other hand, Tanabe Seiyaku Co. Ltd. tested a broad range of entrapping matrices and found best results with polyacrylamide. In the plug-flow process, it was found that suspending the immobilized cells in their substrate for 24 to 28 h increased their activity tenfold. Once immobilized, the cell column was very stable, with a half-life of 120 d at 37°C. After acidification of the effluent from the column, the L-aspartic acid crystallizes and can be collected by centrifugation or filtration. This system has been operated since 1973 [55]. Overall costs compared with the fermentative batch production were reduced by ~40% due to a significant increase in productivity and due to the reduction of labor and wastewater costs. In 1978, the trapping matrix changed to κ-carrageenan, a polysaccharide obtained from seaweed that increased the relative productivity 15-fold. Approximately 100 t/month of L-aspartic acid can be manufactured using a 1000 L reactor. The process was further improved by developing *E. coli* strains with increased aspartase activity. These improved *E. coli* strains are used, since 1982, in the production process at Tanabe Seiyaku Co. Ltd. [56].

BioCatalytics (USA) uses a plug-flow reactor (75 L vol.) with immobilized *E. coli* cells with high aspartase activity on polyurethane and polyazetidene. Space-time yields of 3 kg/L/h are obtained and downstream processing of L-aspartate is performed by acid induced precipitation. A new production system was established in the 1980s by using intact resting cells of coryneform bacteria without immobilization [52]. This genetically engineered bacterial strain possesses high maleate isomerase and aspartase activities. A repetitive batch process using suspended *Corynebacterium glutamicum* (synonym: *Brevibacterium flavum*) cells was started at Mitsubishi Petrochemical Co. Ltd. (Japan), in 1986 [57,58]. The bacterial cells are retained by ultrafiltration.

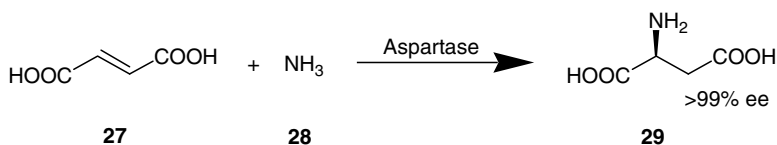


FIGURE 27.7 Synthesis of L-aspartate **29** catalyzed by aspartase.

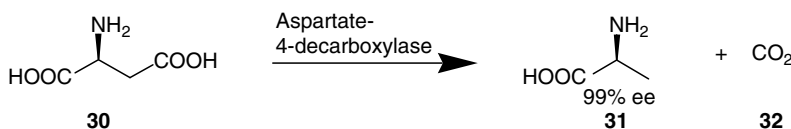


FIGURE 27.8 Decarboxylation of L-aspartate **30** to L-alanine **31** catalyzed by aspartate-4-decarboxylase.

27.3.3 ASPARTATE-4-DECARBOXYLASE

Aspartate-4-decarboxylase (E.C. 4.1.1.12) (synonym: aspartate beta-decarboxylase; systematic name: L-aspartate 4-carboxy-lyase) is a pyridoxal-5'-phosphate dependent enzyme that catalyzes the nonoxidative decarboxylation of L-aspartate **30** to L-alanine **31** [59]. This reaction is used for a one-step batch process using immobilized whole cells of *Pseudomonas dacunhae* at Tanabe Seikyaku Co. Ltd. (Japan) since 1965 (Figure 27.8). The setup of a continuous plug-flow reactor using immobilized cells was first complicated by the evolution of gaseous CO_2 from the decarboxylation reaction, which made plug flow of the substrates difficult and caused significant pH changes. This problem was overcome with the development of a pressurized fixed-bed reactor operating at 10 bar [60]. To improve the yield of L-alanine, the alanine racemase, and fumarase activities can be destroyed in the cells by acid treatment (pH 4.75, 30°C). During this treatment aspartate-4-decarboxylase is stabilized by the addition of pyruvate and pyridoxal-5'-phosphate.

Tanabe Seikyaku Co. Ltd. combined the process with the aspartase catalyzed synthesis of L-aspartic acid from fumarate in a two-step biotransformation [61]. The main reason for the separation of the reaction in two reactors is the difference in pH-optima of both enzymes (aspartase from *E. coli*: pH 8.5, and aspartate-4-decarboxylase from *P. dacunhae*: pH 6.0). Using a 1000 L reactor for immobilized *E. coli* cells and a 2000 L pressurized reactor for *P. dacunhae* cells, about 100 t of L-aspartic acid and 100 t of L-alanine can be produced per month, respectively. Tanabe commercialized this system of a sequential enzyme reaction using two kinds of immobilized microbial cells in 1982 [56].

27.3.4 L-PHENYLALANINE AMMONIA LYASE

L-Phenylalanine ammonia lyase (E.C. 4.3.1.5) (synonyms: PAL, L-phenylalanine deaminase, tyrase; systematic name: L-phenylalanine ammonia lyase) catalyzes the reversible cleavage of ammonia **25** from L-phenylalanine **34** yielding *trans*-cinnamic acid **33** without additional cofactor. The reverse reaction is used in industrial biotransformations to produce L-phenylalanine [62] (Figure 27.9). This amino acid is used in the synthesis of the sweetener aspartame and in parenteral nutrition. Further it is the chiral building block in the synthesis of the macrolide antibiotic rutamycin B [63]. PALs from a broad range of bacteria and eukaryotes have been characterized and the genes were cloned [22].

The Genex Corporation (USA) established a fed-batch process, in 1986, with suspended *Rhodotorula rubra* cells in aqueous medium at pH 10.6 [64]. As PAL from *R. rubra* is

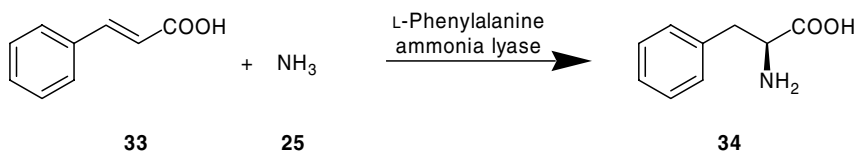


FIGURE 27.9 L-Phenylalanine **34** synthesis catalyzed by L-phenylalanine ammonia lyase.

sensitive to oxygen, the biotransformation is performed under anaerobic, static conditions. The reaction is performed in fed-batch mode with periodical addition of concentrated ammonium cinnamate solution and pH is adjusted by the addition of CO₂ [65]. The fermentative process was improved by starting the process directly from glucose as a substrate and L-phenylalanine is now manufactured by fermentation using overproducing cells on a scale of 8 to 10,000 t/y [16].

27.4 C–O BOND FORMATION

27.4.1 FUMARASE

Fumarase (E.C. 4.2.1.2) (synonyms: fumarate hydratase; systematic name: (*S*)-maleate hydro-lyase) catalyzes the reversible cleavage of water from (*S*)-malate **36** without a cofactor. Fumarases have been identified in a broad range of bacteria and eukaryotes [22]. For industrial production of (*S*)-malate—which is used as an acidulant, in competition with citric acid, in fruit, in vegetable juices, carbonated soft drinks, jams and candies, in amino acid infusions, and in the treatment of hepatic malfunctioning—the enzymes from *C. glutamicum* and *C. ammoniagenes* are applied (Figure 27.10) [66,67]. Approximately 40,000 t of malic acid are used worldwide annually. The process developed by the Amino GmbH uses suspended cells of *C. glutamicum* in a batch reactor [68,69]. The production was started in the year 1988, and (*S*)-malate **36** was produced with an annual productivity of 2000 t. The process is highly enantioselective with no (*R*)-malate detected. The reaction is carried out in a slurry of crystalline calcium fumarate and calcium malate. The process is forced to quantitative conversion of fumarate **35** by *in situ* precipitation of the product shifting the equilibrium toward calcium malate.

27.4.2 MALEASE

Malease (E.C. 4.2.1.31) (synonym: maleate hydratase; systematic name: (*R*)-maleate hydro-lyase). Maleases have been found not only in many bacteria, yeasts, and fungi but also in plants and animals. However, the protein chemical and enzymological data known so far suggest that malease activity is catalyzed by different types of enzymes. So far, there are no sequence data, neither of the protein nor of the DNA-sequence available [22]. *P. pseudoalcaligenes*, which is used in a commercial process of DSM Ltd. (NL) for the production of (*R*)-malic acid **39** (Figure 27.11) was identified from a thorough microbial screening [70]. (*R*)-Malic acid is an optically active 2-hydroxy acid that can be used as a chiral synthon, as a resolving agent, or as a ligand in asymmetric synthesis [71,72]. Malease from *P. pseudoalcaligenes* is a hetero dimeric enzyme that requires no additional cofactors for catalysis. The enzyme is used in a batch process with immobilized whole cells in aqueous medium. The cheaper maleic anhydride **37** can be used instead of maleic acid **38**, as it hydrolyzes *in situ* to the substrate. Optimization of the process is still a matter of research [73].

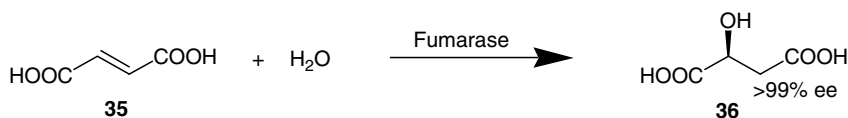


FIGURE 27.10 Addition of water to fumarate **35** yielding L-malate **36** catalyzed by fumarase.

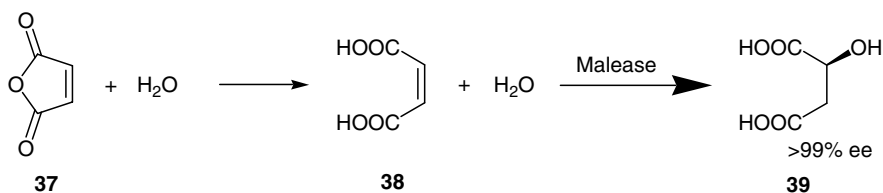


FIGURE 27.11 Production of (*R*)-malic **39** acid catalyzed by malease.

27.4.3 NITRILE HYDRATASE

Nitrile hydratases (E.C. 4.2.1.84) (systematic name: nitrile hydro-lyase) catalyze the reversible cleavage of water from aliphatic amides. The enzyme has been found in different bacteria and structural data are available [22].

Nitrile hydratases belonging to the enzyme class of lyases (E.C. 4) should not be confused with the nitrilases belonging to the class of hydrolases (E.C. 3) that hydrolyze nitriles to the corresponding carboxylic acids. For technical purposes, the enzymes from *Pseudomonas chlororaphis* and *Rhodococcus rhodochrous* are used as immobilized whole cells to produce various amides by the addition of water to the respective nitriles.

DuPont uses the strain *P. chlororaphis* B23 to synthesize 5-cyano valeramide **41** (Figure 27.12), which is used as an intermediate for the synthesis of the herbicide azafenidine [74]. Whole cells from *P. chlororaphis* are immobilized in calcium alginate beads. For strain selection, it was important that the cells did not show any amidase activity that would further hydrolyze the amide to the carboxylic acid. The biotransformation is carried out in a two-phase system with pure adiponitrile **40** forming the organic phase [74]. A reaction temperature of 5°C is chosen, as the solubility of the by-product adipodiamide is only 37 to 42 mM in 1 to 1.5 M 5-cyanovaleramide **41**. A batch reactor is preferred over a fixed-bed reactor, because of the lower selectivity toward 5-cyanovaleramide that was observed, the possibility of precipitation of adipodiamide, and plugging of the column. Excess water is removed by distillation at the end of the reaction. The by-product adipodiamide is precipitated by dissolution of the resulting oil in methanol at >65°C. The crude product solution is directly transferred to the herbicide synthesis.

Using this method, 13.6 t have been produced in 58 repetitive batch cycles with 97% conversion and 96% selectivity. This biotransformation was chosen over the chemical transformation because of the higher conversion and selectivity, production of more product per catalyst weight (3150 kg/kg dry cell weight), and less waste. The catalyst consumption is 0.006 kg/kg product.

Acrylamide **43** (Figure 27.13) is an important commodity monomer used in coagulants, soil conditioners, and stock additives for paper treatment and paper sizing, for adhesives, paints, and petroleum recovering agents. It is biocatalytically produced by Nitto Chemical Industry Co. Ltd. (Japan), using *R. rhodochrous* as a production strain that was selected out of 1000 microbial strains [75–79].

Acrylamide **43** is unstable and polymerizes easily; therefore the process is carried out at low temperature (5°C). Although the cells are immobilized on polyacrylamide gel and

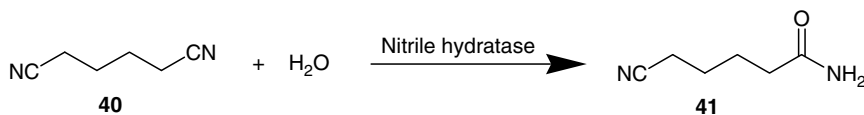


FIGURE 27.12 Synthesis of 5-cyano valeramide **41** catalyzed by nitrile hydratase.

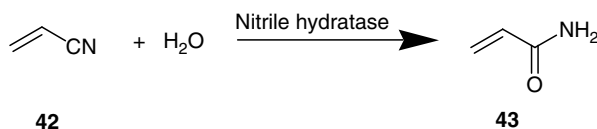


FIGURE 27.13 Synthesis of acrylamide **43** catalyzed by nitrile hydratase.

the containing enzymes are very stable toward acrylonitrile **42**, the starting material has to be fed continuously to the reaction mixture because of the inhibition effects at higher concentrations. The biotransformation is started with an acrylonitrile concentration of 0.11 M and is stopped at 5.6 M. The process is operated at a capacity of 30,000 t/y.

The chemical synthesis uses copper salt as a catalyst for the hydration of acrylonitrile and has the following disadvantages:

- The rate of acrylamide formation is lower than that of acrylic acid formation.
- The double bond of the starting material and the product causes the formation of by-products such as ethylene, cyanohydrin, and nitrilotrispropionamide.
- Polymerization occurs.
- Copper needs to be separated from the product (an extra step in the chemical synthesis).

Biotransformation has the advantage of 100% conversion of the nitrile, thereby avoiding recovering nonreacted nitrile and removing the copper catalyst required in the chemical process. This is also the first case of a biocatalytic conversion of a bulk fiber monomer.

Nitrile hydratase from *R. rhodochrous* also acts on other nitriles with yields of 100%. The most impressive example is the conversion of 3-cyanopyridine **44** to nicotinamide **45**, a biotransformation by Lonza AG (production site China) [80]. Nicotinamide (vitamin B3) is used as a vitamin supplement for food and animal feed. The biotransformation (a continuous process) is operated at low temperature and atmospheric pressure and is carried out on a scale of 3000 t/y (Figure 27.14).

The product concentration is about 1465 g/L. This conversion (1.17 g/L dry cell mass) can be named “pseudocrystal enzymation,” because the substrate is solid at the beginning of the reaction and dissolved with ongoing reaction [80]. In contrast to the chemical alkaline hydrolysis of 3-cyanopyridine where nicotinic acid (4%) is formed as a by-product, the biotransformation is absolutely selective and no acid or base is required. Compared to the old synthetic route of nicotinamide at Lonza, the new one is environmentally friendly and safe. There is only one organic solvent used throughout the whole process in four highly selective continuous and catalytic reaction steps.

27.4.4 CARNITINE DEHYDRATASE

Carnitine dehydratases (E.C. 4.2.1.89) (systematic name: L-carnitine hydrolyase) have been described in only a few microorganisms. The enzyme catalyzes the reversible cleavage

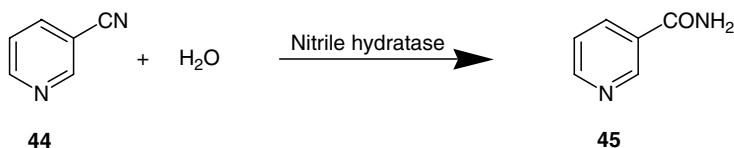


FIGURE 27.14 Synthesis of nicotinamide **45** catalyzed by nitrile hydratase.

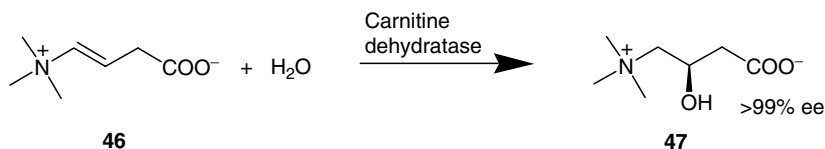


FIGURE 27.15 Synthesis of L-carnitine **47** catalyzed by carnitine dehydratase.

of water from L-carnitine **47** to 4-(trimethylamino)butanoate **46** and the reverse reaction is used for the production of L-carnitine (Figure 27.15). L-Carnitine facilitates the transport of fatty acids across mitochondrial membrane, and thus is used as a nutritional supplement ingredient, especially in sports and health beverages. Only the L-isomer is biologically active, which gives stereoselective enzyme-based processes an advantage over chemical synthesis [81]. Carnitine dehydratase from *E. coli* was thoroughly characterized and the gene was cloned [22].

A good example is the manufacture of L-carnitine from butyrobetaine through 4-butyrobetaine CoA by Lonza AG (Visp, Switzerland) [82,83]. This process involves site-selective reaction of a nonactivated carbon atom. What is interesting is that L-carnitine has been a product, for a sufficiently long period, for the butyrobetaine process to be a second-generation bioprocess, replacing the original bioprocess that was based on the use of chloroacetoacetic acid ester as the precursor. Furthermore, Lonza has described how long it took them to develop a high-yielding process (300 g/L) for butyrobetaine conversion, with further improvements becoming increasingly difficult to achieve as the concentrations of the product became gradually higher. This involved improving the original low-yielding microbial strain to a production strain that produces about 2000-fold higher concentration of carnitine. This was achieved by avoiding the metabolism of L-carnitine with strains lacking in carnitine dehydrogenase [82]. 4-Butyrobetainyl coenzyme A is the key intermediate in the metabolism. An aspect of the microorganisms physiology that greatly improves the productivity of the process is that butyrobetaine uptake is mediated by a periplasmic adenosine triphosphate-linked protein, which is closely coupled to carnitine excretion. This means that more butyrobetaine substrate is taken up by the cell for every molecule of carnitine exported. Therefore, alternative mutants of the original strain are now used for production and Lonza has achieved a 103-fold increase in activity from the original mutant after 1.5 years of work.

27.5 CONCLUSION

Presently, biocatalytic C–C, C–N, and C–O formations play an important role in industrial syntheses of advanced intermediates. Often, new chiral centers are formed starting from achiral compounds like aldehydes. As there is no kinetic resolution involved, a maximum theoretical yield of 100%, is possible. In future, new biocatalytic activities will be available by molecular engineering and directed evolution. Lyases have a bright future in industrial applications for preparation of fine chemicals and chiral intermediates.

REFERENCES

1. Liese, A., Seelbach, K., and Wandrey, C., *Industrial Biotransformations*, 2nd ed., Wiley-VCH, Weinheim, Germany, 2006.
2. Breuer, M., Ditrich, K., Habicher, T., et al., Industrial methods for the production of optically active intermediates, *Angew. Chem. Int. Ed. Engl.*, 43, 788–824, 2004.

3. Straathoff, A.J.J., Panke, S., Schmid, A., The production of fine chemicals by biotransformations, *Curr. Opin. Biotechnol.*, 13, 548–556, 2002.
4. Panke, S. and Wubbolts, M., Advances in biocatalytic synthesis of pharmaceutical intermediates, *Curr. Opin. Chem. Biol.*, 9, 188–194, 2005.
5. Rosenthaler, L., Asymmetric syntheses produced by enzymes, *Biochem. Z.*, 14, 238–253, 1909.
6. Rosche, B., Breuer, M., Hauer, B., et al., Screening of yeasts for cell-free production of (*R*)-phenylacetylcarbinol, *Biotechnol. Lett.*, 25, 841–845, 2003.
7. Goetz, G., Iwan, P., Hauer, B., et al., Continuous production of (*R*)-phenylacetylcarbinol in an enzyme-membrane reactor using a potent mutant of pyruvate decarboxylase from *Zymomonas mobilis*, *Biotechnol. Bioeng.*, 74, 317–325, 2001.
8. Leksawasdi, N., Chow, Y.Y.S., Breuer, M., et al., Kinetic analysis and modelling of enzymatic (*R*)-phenylacetylcarbinol batch biotransformation process, *J. Biotechnol.*, 111, 179–189, 2004.
9. Shukla, V.B. and Kulkarni, P.R., Comparative studies on bioconversion of benzaldehyde to L-phenylacetylcarbinol (L-PAC) using calcium alginate- and barium alginate-immobilized cells of *Torulaspora delbrueckii*, *J. Chem. Technol. Biotechnol.*, 78, 949–951, 2003.
10. Shukla, V.B. and Kulkarni, P.R., L-Phenylacetylcarbinol (L-PAC): biosynthesis and industrial applications, *World J. Microbiol. Biotechnol.*, 16, 499–506, 2000.
11. Rosche, B., Leksawasdi, N., Sandford, V., et al., Enzymatic (*R*)-phenylacetylcarbinol production in benzaldehyde emulsions, *Appl. Microbiol. Biotechnol.*, 60, 94–100, 2002.
12. Rosche, B., Sandford, V., Breuer, M., et al., Enhanced production of *R*-phenylacetylcarbinol (*R*-PAC) through enzymatic biotransformation, *J. Mol. Catal., B Enzym.*, 19, 109–115, 2002.
13. Oliver, A.L., Anderson, B.N., Roddick, F.A., Factors affecting the production of L-phenylacetylcarbinol by yeast: a case study, *Adv. Microb. Physiol.*, 41, 1–45, 1999.
14. Pohl, M., Sprenger, G.A., Müller, M., A new perspective on thiamine catalysis, *Curr. Opin. Biotechnol.*, 15, 335–342, 2004.
15. Ward, O.P. and Singh, A., Enzymatic asymmetric synthesis by decarboxylases, *Curr. Opin. Biotechnol.*, 11, 520–526, 2000.
16. Breuer, M. and Hauer, B., Carbon–carbon coupling in biotransformation, *Curr. Opin. Biotechnol.*, 14, 570–576, 2003.
17. Siegert, P., McLeish, M.J., Baumann, M., et al., Exchanging the substrate specificities of pyruvate decarboxylase from *Zymomonas mobilis* and benzoylformate decarboxylase from *Pseudomonas putida*, *Protein Eng. Des. Sel.* 18, 345–357, 2005.
18. Müller, M., Chemoenzymatic synthesis of building blocks for statine side chains, *Angew. Chem. Int. Ed. Engl.*, 44, 362–365, 2005.
19. Kierkels, J.G.T., Mink, D., Panke, S., et al., Process for the preparation of 2,4-dideoxyhexoses and therapeutic uses thereof, WO 2003/006656 (DSM).
20. DeSantis, G., Liu, J., Clark, D.P., et al., Structure based mutagenesis approaches towards expanding the substrate specificity of D-2-deoxyribose-5-phosphate aldolase, *Bioorg. Med. Chem.*, 11, 43–52, 2003.
21. Liu, J., Hsu, C.-C., and Wong, C.-H., Sequential aldol condensation catalyzed by DERA mutant Ser238Asp and a formal total synthesis of atorvastatin, *Tetrahedron Lett.*, 45, 2439–2441, 2004.
22. BRENDA Enzyme Database at <http://www.brenda.uni-koeln.de/>.
23. Ohta, Y., Tsukada, Y., Sugimori, T., et al., Isolation of a constitutive *N*-acetylneuraminate lyase-producing mutant of *Escherichia coli* and its use for NPL production, *Agric. Biol. Chem.*, 53, 477–481, 1989.
24. Maru, I., Ohnishi, J., Ohta, Y., et al., Simple and large-scale production of *N*-acetylneuraminic acid from *N*-acetyl-D-glucosamine and pyruvate using *N*-acetyl-D-glucosamine 2-epimerase and *N*-acetylneuraminate lyase, *Carbohydr. Res.*, 306, 575–578, 1998.
25. Dawson, M., Noble, D., and Mahmoudia, D.S., Process for the preparation of *N*-acetyl-D-neuraminic acid, PCT WO 9429476, 1994.
26. Mahmoudian, M., Noble, D., Drake, C.S., et al., An efficient process for production of *N*-acetylneuraminic acid using *N*-acetylneuraminic acid aldolase, *Enzyme Microb. Technol.*, 45, 393–400, 1997.

27. Ghisalba, O., Gygax, D., Kragl, U., et al., Enzymatic method for *N*-acetyneuramin acid production, Novartis, EP 0428947, 1991.
28. Kragl, U., Gygax, D., Ghisalba, O., et al., Enzymatic process for preparing *N*-acetyneuraminic acid, *Angew. Chem. Int. Ed. Engl.*, 30, 827–828, 1991.
29. Bang, W., Lang, S., Sahm, H., et al., Production of L-tryptophan by *Escherichia coli* cells, *Biotechnol. Bioeng.*, 25, 999–1011, 1983.
30. Plischke, H. and Steinmetzer, W., Verfahren zur Herstellung von L-Tryptophan und D,L-Serin, AMINO GmbH, DE 3630878 C1, 1988.
31. Sharma, M., Sharma, N.N., and Bhalla, T.C., Hydroxynitrile lyases: at the interface of biology and chemistry, *Enzyme Microb. Technol.*, 37, 279–294, 2005.
32. Gruber, K. and Kratky, Ch., Biopolymers for biocatalysis: structure and catalytic mechanism of hydroxynitrile lyases, *J. Polym. Sci. [A]*, 42, 479–486, 2004.
33. Effenberger, F., Synthesis and reactions of optically active cyanohydrins, *Angew. Chem. Int. Ed. Engl.*, 33, 1555, 1994.
34. Effenberger, F., Optically active cyanohydrins—important sources for chiral drugs, *Enantiomer*, 1, 359, 1996.
35. Effenberger, F., Enzyme-catalyzed preparation and synthetic applications of optically active cyanohydrins, *Chimia.*, 53, 3, 1999.
36. Griengl, H., Hickel, A., Johnson, D.V., et al., Enzymatic cleavage and formation of cyanohydrins: a reaction of biological and synthetic relevance, *Chem. Commun.*, 20, 1933–1940, 1997.
37. Johnson, D.V., Zabelinskaja-Mackova, A.A., and Griengl, H., Oxynitrilases for asymmetric C–C bond formation, *Curr. Opin. Chem. Biol.*, 4, 103, 2004.
38. Griengl, H., Schwab, H., and Fechter, M., The synthesis of chiral cyanohydrins by oxynitrilases, *Ophthalmic Genet.*, 18, 252, 2000.
39. Glieder, A., Weis, R., Skranc, W., et al., Comprehensive step-by-step engineering of an (*R*)-hydroxynitrile lyase for large-scale asymmetric synthesis, *Angew. Chem. Int. Ed. Engl.*, 42, 4815–4818, 2003.
40. Chemie Linz GmbH: DNA encoding *Hevea brasiliensis* (*S*)-hydroxy:nitrilase, DE 195229116, *Chem. Abstr.*, 126, 208953, 1997.
41. Semba, H. (Nippon Shokubai): Process for producing *S*-hydroxynitrile lyase, EP 1016712, *Chem. Abstr.*, 133, 85120, 2000.
42. Effenberger, F., Wajant, H., Lauble, P., et al. (Fine Chemicals Austria): (*S*)-Hydroxynitrile lyases with improved substrate acceptance and uses thereof, EP 969095, *Chem. Abstr.*, 132, 191223, 2000.
43. Bousquet, A. and Musolino, A. (Sanofi-Synthelabo): Hydroxyacetic ester derivatives, preparation method and use as synthesis intermediates, EP 1021449, *Chem. Abstr.*, 130, 296510, 1999.
44. Pöchlauer, P. and Mayrhofer, H. (DSM Fine Chemicals Austria): Method for producing optically and chemically pure (*R*)- and (*S*)-hydroxycarboxylic acids, EP 1148042, *Chem. Abstr.*, 135, 303603, 2001.
45. Okuda, N., Semba, H., and Dobashi, Y. (Nippon Shokubai): A method for producing alpha-hydroxycarboxylic acid, EP 1160235, *Chem. Abstr.*, 136, 5721, 2002.
46. Semba, H. and Dobashi, Y. (Nippon Shokubai): An enzyme reaction method and a method for enzymatically producing an optically active cyanohydrin, EP 1160329, *Chem. Abstr.*, 136, 4802, 2002.
47. Hasslacher, M., Schall, M., Schwab, H., et al. (DSM Chemie Linz): (*S*)-Hydroxynitrilase from *Hevea brasiliensis*, WO 97/03204, *Chem. Abstr.*, 126, 196118, 2000.
48. Griengl, H., Klempier, N., Pöchlauer, P., et al., Enzyme catalyzed formation of (*S*)-cyanohydrins derived from aldehydes and ketones in a biphasic solvent system, *Tetrahedron*, 54, 1447, 1998.
49. Semba, H. (Nippon Shokubai): Method for the production of optically active cyanohydrins, EP 1026256, *Chem. Abstr.*, 133, 134248, 2000.
50. Pöchlauer, P., Schmidt, M., Wirth, I., et al. (DSM Fine Chemicals Austria): Enzymatic process for the preparation of (*S*)-cyanohydrins, EP 927766, *Chem. Abstr.*, 131, 72766, 2000.
51. Weis, R., Gaisberger, R., Skranc, W., et al., Carving the active site of almond R-HNL for increased enantioselectivity, *Angew. Chem. Int. Ed. Engl.*, 44, 4700–4704, 2005.
52. Kumagai, H., Microbial production of amino acids in Japan, in *Advances in Biochemical Engineering and Biotechnology*, vol. 69, Schepers, T., Ed., Springer, Berlin/Heidelberg, 2000, pp. 71–85.

53. Tsuchida, T., Nishimoto, Y., Kotani, T., et al., Production of L-3,4-dihydroxyphenylalanine, Ajinomoto Co. Ltd., JP 5123177A, 1993.
54. Yamada, H., Screening of novel enzymes for the production of useful compounds, in *New Frontiers in Screening for Microbial Biocatalysis*, Kieslich, K., van der Beek, C.P., de Bont, J.A.M., et al., Eds., *Studies in Organic Chemistry*, Elsevier, Amsterdam, 1998, 53, pp. 13–17.
55. Tanaka, A., Tosa, T., and Kobayashi, T., *Industrial Application of Immobilized Biocatalysts*, Marcel Dekker, New York, 1993.
56. OECD. The application of biotransformation to industrial sustainability, ISBN 92-64-1946-7 No 52184 200, OECD Publications, France, 2001.
57. Terasawa, M., Yukawa, H., and Takayama, Y., Production of L-aspartic acid from *Brevibacterium* by cell re-using process, *Process Biochem.*, 20, 124–128, 1985.
58. Yamagata, H., Teresawa, M., and Yukawa, H., A novel industrial process for L-aspartic acid production using an ultrafiltration-membrane, *Catal. Today*, 22, 621–627, 1994.
59. Schmidt-Kastner, G. and Egerer, P., Amino acids and peptides, in *Biotechnology*, vol. 6a, Kieslich, K., Ed., Verlag Chemie, Weinheim, 1984, pp. 387–419.
60. Furui, M. and Yamashita, K., Pressurized reaction method for continuous production of L-alanine by immobilized *Pseudomonas dacunhae* cells, *J. Ferment. Technol.*, 61, 587–591, 1983.
61. Takamatsu, S., Umemura, J., Yamamoto, K., et al., Production of L-alanine from ammonium fumarate using two immobilized microorganisms, *Eur. J. Appl. Biotechnol.*, 15, 147–152, 1982.
62. Sheldon, R.A., *Chirotechnology*, Marcel Dekker, New York, 1993.
63. Cheetham, P.S.J., Bioprocess for the manufacture of ingredients for food and cosmetics, *Adv. Biochem. Eng. Biotechnol.*, 86, 83–158, 2004.
64. Vollmer, P.J., Montgomery, J.P., Schrufer, J.J., et al., Method for stabilizing the enzymatic activity of phenylalanine ammonia lyase during L-phenylalanine production, Genex Corporation, US 4584269, 1985.
65. Crosby, J., Synthesis of optically active compounds: a large scale perspective, *Tetrahedron*, 47, 4789–4846, 1991.
66. Matthey, M., The production of organic acids, *Crit. Rev. Biotechnol.*, 12, 87–132, 1988.
67. Tosa, T. and Shibatani, T., Industrial applications of immobilized biocatalysts in Japan, *Ann. N. Y. Acad. Sci.*, 750, 364–375, 1995.
68. Daneel, H.J., Busse, M., and Faurie, R., Pharmaceutical grade L-malic acid from fumaric acid—development of an integrated biotransformation and product purification process, *Med. Fac. Landbouww. Univ. Gent.*, 60(4a), 2093–2096, 1995.
69. Daneel, H.J., Busse, M., and Faurie, R., Fumarate hydratase from *Corynebacterium glutamicum*—process related optimization of enzyme productivity for biotechnical L-malic acid synthesis, *Med. Fac. Landbouww. Univ. Gent.*, 61(4a), 1333–1340, 1996.
70. Van der Werf, M., van der Tweel, W., and Hermans, S., Screening for microorganisms producing D-malate from maleate, *Appl. Environ. Microbiol.*, 58, 2854–2860, 1992.
71. Chibata, I., Tosa, T., and Shibatani, T., The industrial production of optically active compounds by immobilized biocatalysts, in *Chirality in Industry*, Collins, A.N., Sheldrake, G., and Crosby, J., Eds., Wiley, New York, 1992, pp. 351–370.
72. Submarmarian, S.S. and Rao, R.M.R., Purification and properties of citraconase, *J. Biol. Chem.*, 243, 2367–2372, 1968.
73. Michielsen, M., Frielink, C., Wijffels, R., et al., D-Malate production by permeabilized *Pseudomonas speudoalcaligenes*: optimization of conversion and biocatalyst productivity, *J. Biotechnol.*, 79, 13–26, 2000.
74. Hann, E.C., Eisenberg, A., Fager, S.K., et al., 5-Cyanovaleramide production using immobilized *Pseudomonas chlororaphis* B23, *Bioorg. Med. Chem.*, 7, 2239–2245, 1999.
75. Nagasawa, T., Shimizu, H., and Yamada, H., The superiority of the third-generation catalyst, *Rhodococcus rhodochrous* J1 nitrile hydratase, for industrial production of acrylamide, *Appl. Microb. Biotechnol.*, 40, 189–195, 1993.
76. Shimizu, H., Ogawa, J., Kataoka, M., et al., Screening of novel microbial enzymes for the production of biologically and chemically useful compounds, in *New Enzymes for Organic Synthesis*,

- Ghose, T.K., Fiechter, A., and Blakebrough, N., Eds., Springer, Berlin/Heidelberg, 1997; *Adv. Biochem. Eng. Biotechnol.*, 58, 56–59, 1997.
77. Yamada, H. and Tani, Y., Process for biologically producing amide, EP 093782, 1982.
78. Yamada, H. and Kobayashi, M., Nitrile hydratase and its application to industrial production of acrylamide, *Biosci. Biotech. Biochem.*, 60(9), 1391–1400, 1996.
79. Yamada, H. and Tani, Y. (Nitto Chemical Industry Co. Ltd.): Process for biological preparation of amides. US 4637982, 1987.
80. Petersen, M. and Kiener, A., Biocatalysis—preparation and functionalization of *N*-heterocycles, *Green Chem.*, 4, 99–106, 1999.
81. Kulla, H., Enzymatic hydroxylations in industrial application, *Chimica* 45, 81–85, 1991.
82. Zimmermann, Th.P., Robins, K.T., Werlen, J., et al., Bio-transformation in the production of L-carnitine, in *Chirality in Industry*, Collins, A.N., Sheldrake, G.N., and Crosby, J., Eds., Wiley, New York, 1997, pp. 287–305.
83. Hoeks, F.W.J.M.M., Verfahren zur diskontinuierlichen Herstellung von L-Carnitin auf mikrobiologischem Weg. Lonza AG, EP 410 430 A2, 1991.

28 State of the Art and Applications in Stereoselective Synthesis of Chiral Cyanohydrins

*Franz Effenberger, Siegfried Förster,
and Christoph Kobler*

CONTENTS

28.1	Introduction	678
28.2	Metal-Catalyzed Syntheses of Chiral Cyanohydrins	678
28.2.1	Application of Chiral Bidentate and Tridentate Ligands	678
28.2.2	Application of Chiral Tetradentate Ligands	679
28.2.3	Summary of Metal-Catalyzed Synthesis of Chiral Cyanohydrins	680
28.3	Enzyme-Catalyzed Preparations of Chiral Cyanohydrins	680
28.3.1	Optimization of Reaction Conditions for HNL-Catalyzed Cyanohydrin Formation	680
28.3.2	(<i>R</i>)-HNL Catalysis	681
28.3.3	(<i>S</i>)-HNL Catalysis	682
28.3.4	Lipase-Catalyzed Preparation of Chiral Cyanohydrins	683
28.3.5	Summary of Enzyme-Catalyzed Preparations of Chiral Cyanohydrins	684
28.4	Crystal Structures of HNLs and Mechanism of Cyanogenesis	684
28.4.1	Crystal Structures of HNLs	684
28.4.2	Reaction Mechanism of Cyanogenesis	685
28.4.3	Changing Substrate Specificity and Stereoselectivity by Applying Mutants of MEHNL	687
28.4.4	Summary of Crystal Structures of HNLs and Mechanism of Cyanogenesis	687
28.5	Important Applications of HNLs in Organic Synthesis	687
28.5.1	Biologically Active Cyanohydrin Esters	688
28.5.2	Chiral 2-Hydroxy Acids	688
28.5.3	Nonracemic 1,2-Amino Alcohols	690
28.5.4	Cyanohydrins of Substituted Cyclohexanones	691
28.6	Conclusion	693
	References	693

28.1 INTRODUCTION

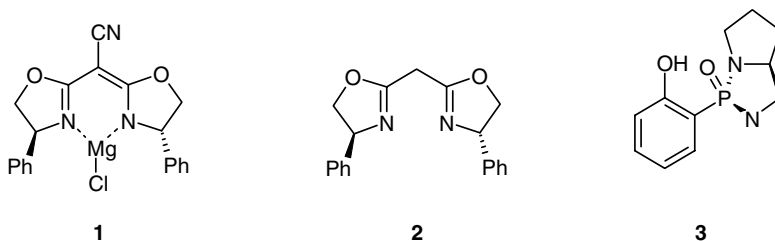
Michael North, guest editor of the *Tetrahedron Symposium in Print* Number 109, has summarized in his preface the most important actual research activities on the synthesis and applications of nonracemic (chiral) cyanohydrins [1]. Since the beginning of remarkable research activities on chiral cyanohydrins in 1987 [2], several excellent review articles on this topic have been published [3–12]. For this reason, this chapter only discusses the developments of the last 5 y in synthesis and applications of nonracemic cyanohydrins with special emphasis on applications of hydroxynitrile lyases (HNLs) as chiral catalysts.

28.2 METAL-CATALYZED SYNTHESIS OF CHIRAL CYANOHYDRINS

Although HNL-catalyzed preparations of nonracemic cyanohydrins are still the most important synthetic approach to chiral cyanohydrins, metal-catalyzed reactions have received considerable interest during the last decade [13,14]. The discovery and development of new efficient catalysts, containing a metal and chiral ligands, has resulted in much higher enantioselectivities of the cyanohydrins prepared. Since enzyme-catalyzed reactions depend strongly on structural requirements of the reacting substrates, metal-catalyzed cyanohydrin formations could become important and interesting in cases of sterically demanding aldehydes and especially in the reactions of ketones [13,14]. North classifies the various metal catalysts according to the nature of the chiral ligands [13].

28.2.1 APPLICATION OF CHIRAL BIDENTATE AND TRIDENTATE LIGANDS

Corey and Wang have generated a catalytically active system for the asymmetric addition of trimethylsilyl cyanide (TMSCN) to aldehydes (Scheme 28.1) by using a combination of magnesium bisoxazoline complex **1** as a chiral Lewis acid and uncomplexed bisoxazoline **2** as a chiral Lewis base [15]. The best results (63 to 95% enantiomeric excesses (ee)) were obtained with aliphatic and α,β -unsaturated aldehydes [15]. In a recent publication, chiral oxazaborolidinium salts were efficiently applied to the enantioselective cyanosilylation of ketones [16].

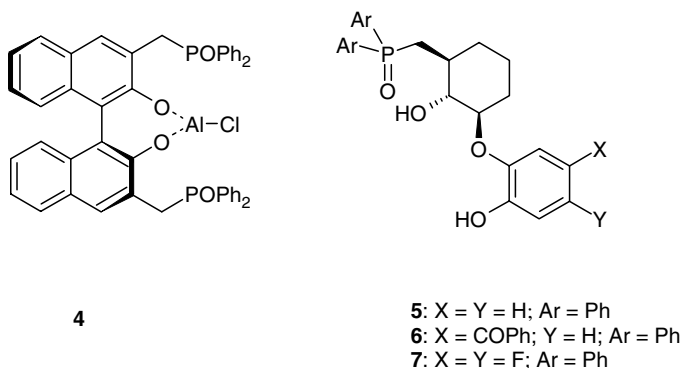


SCHEME 28.1 Bidentate ligands for asymmetric cyanosilylation.

For the addition of TMSCN to aromatic aldehydes, Brunel et al. were able to obtain ee up to 98% by using a titanium complex of diastereomerically pure phosphine oxide **3** (Scheme 28.1) [17].

BiNOL and its derivatives have also been used successfully as bidentate chiral ligands for the enantioselective addition of TMSCN to aldehydes and ketones, respectively [13]. The most efficient BiNOL complex in asymmetric cyanohydrin synthesis is the Al complex of BiNOL derivative **4** (Scheme 28.2) [18]. The best results (up to 98% ee) are obtained in the presence of an additional phosphine oxide additive [18].

Hamashima et al. successfully developed tridentate ligands **5–7** for the enantioselective addition of TMSCN to carbonyl compounds (Scheme 28.2) [19,20]. Although the Al complex of ligand **5** shows no catalytic activity, the corresponding titanium complex was found to be a good catalyst especially for the synthesis of (*R*)-ketone cyanohydrins [19,20].

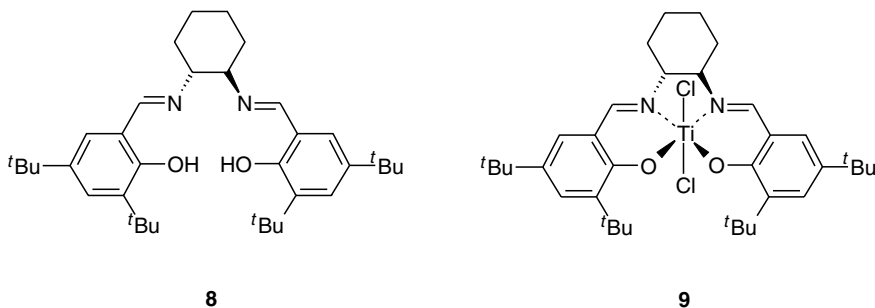


SCHEME 28.2 Bidentate BiNOL derivatives and tridentate ligands for asymmetric cyanosilylation

Interestingly, it was possible to invert the stereochemistry of the TMSCN addition by substitution of titanium by gadolinium [21–23]. Several methyl and ethyl ketones were converted into (*S*)-cyanohydrin trimethylsilyl ethers with 62 to 92% ee. Aryl cycloalkyl ketones also react with high chemical (100%) and optical (95% ee) yields to form the corresponding (*S*)-cyanohydrin trimethylsilyl ethers [21–23].

28.2.2 APPLICATION OF CHIRAL TETRADENTATE LIGANDS

Schiff bases derived from salicylic aldehydes are widely and successfully used chiral tetradentate salen ligands with chiral 1,2-diamines. Interactive studies by the groups of Belokon and North showed ligand **8** to be one of the most promising compounds (Scheme 28.3) [24–29]. The titanium complex **9** is isolated as crystalline solid by the treatment of ligand **8** with TiCl_4 (Scheme 28.3).



SCHEME 28.3 Tetradentate ligands for cyanohydrin synthesis. (From Tararov, V.I., et al., *Chem Commun.*, 387, 1998; Belokon, Y.N., et al., *J. Am. Chem. Soc.*, 121, 3968, 1999; Belokon, Y.N., et al., *Chem Commun.*, 244, 2002; Belokon, Y.N., et al., *Tetrahedron*, 60, 10433, 2004; Chen, F.-X., et al., *Chem Eur. J.*, 10, 4790, 2004.)

Only 0.1 mol% of **9** was needed to convert benzaldehyde to (*S*)-mandelonitrile trimethylsilyl ether with 86% ee [25]. Under the reaction conditions, with traces of water present, complex **9** reacts to give a bimetallic complex that can be isolated and characterized [26]. The bimetallic complex allows the use of potassium cyanide instead of TMSCN as a cyanide source, which is highly advantageous for practical applications. Reactions using KCN are carried out in the presence of an acid anhydride and lead to (*S*)-cyanohydrin carboxylates with 85 to 93% ee [27]. Recently, it could be shown that ethyl cyanofornate can also serve as a cyanide source.

(*R*)-Cyanohydrin ethyl carbonates are obtained in high optical yields (up to 99% ee) when the bimetallic titanium complex [(salen)TiO₂]₂ is used as a chiral catalyst [28]. Cyanosilylation of aromatic as well as aliphatic ketones occurs with high enantioselectivity when a chiral salen-Al complex (2 mol%) and a chiral tertiary amine N-oxide (1 mol%) are combined [29].

Holmes and Kagan have shown that it is not necessary for complex **8** with a transition metal to obtain an effective catalyst for asymmetric cyanohydrin formation: the monolithium salt of **8** catalyzes the asymmetric addition of TMSCN to aldehydes, giving (*R*)-cyanohydrin trimethylsilyl ethers with up to 97% ee [30]. The amount of water present has a significant effect on the enantioselectivity of the Li-salts of complex **8** [31]. Recently, a highly enantioselective cyanosilylation of aldehydes that was catalyzed by novel alcohol–titanium complexes was reported [32].

Polymeric salen–Ti (IV) or –V (V) complexes were employed in the enantioselective *O*-acetyl cyanation of aldehydes with KCN and acetic anhydride [33].

28.2.3 SUMMARY OF METAL-CATALYZED SYNTHESIS OF CHIRAL CYANOHRINS

The metal-catalyzed asymmetric addition of TMSCN to aldehydes and ketones has seen enormous developments during the last decade [13,14]. In particular, the possibility of using potassium cyanide instead of TMSCN as a cyanide source is an important improvement for practical applications of this methodology. Simultaneously, enormous progress has been made in the enzyme-catalyzed synthesis of nonracemic cyanohydrins, especially in HNL-catalyzed reactions. Metal-catalyzed additions will be superior to enzyme catalysis only when the starting carbonyl compounds are not accepted as substrates by the enzymes. This will be the case for sterically demanding aldehydes and especially, ketones.

28.3 ENZYME-CATALYZED PREPARATIONS OF CHIRAL CYANOHRINS

Although considerable progress has been made in metal-catalyzed synthesis of nonracemic cyanohydrins during the last 5 y (see Chapter 27), the HNL-catalyzed preparation of chiral cyanohydrins is still the most important method, especially for large-scale reactions. Progress in lipase-catalyzed kinetic resolution of racemic cyanohydrins and cyanohydrin derivatives will also be discussed, respectively. The kinetic resolution of racemic cyanohydrins by nitrilases and amidases [34] will not be discussed in this chapter.

28.3.1 OPTIMIZATION OF REACTION CONDITIONS FOR HNL-CATALYZED CYANOHRIN FORMATION

All applications of HNLs in catalysis of nonracemic cyanohydrin formation aim to achieve the following objectives: first, it is crucial to get high enantioselectivity [2], suppressing the chemical addition of HCN to the carbonyl substrates. Second, for industrial applications, fast kinetics are necessary for efficient and economical large-scale production. Third, *in situ* formation of HCN is desirable to avoid free handling of toxic HCN.

All the three objectives can be achieved by using the enzymes on a solid support (mainly cellulose) in an organic solvent (diisopropylether and methyl-*tert*-butylether), by applying aqueous media at low pH, or by working in a biphasic system (methyl-*tert*-butylether/water). A comparison of advantages and disadvantages of the various reaction conditions mentioned here can be found in the comprehensive reviews by North [4] and Gröger [35]. For large-scale productions using inexpensive enzymes, the two-phase system is usually the method of choice. In contrast, reactions in organic solvents, with HNLs adsorbed on a support, require considerably smaller amounts of enzymes, and product recovery is facilitated compared to the two-phase system. An interesting alternative to the adsorption of HNLs on cellulose

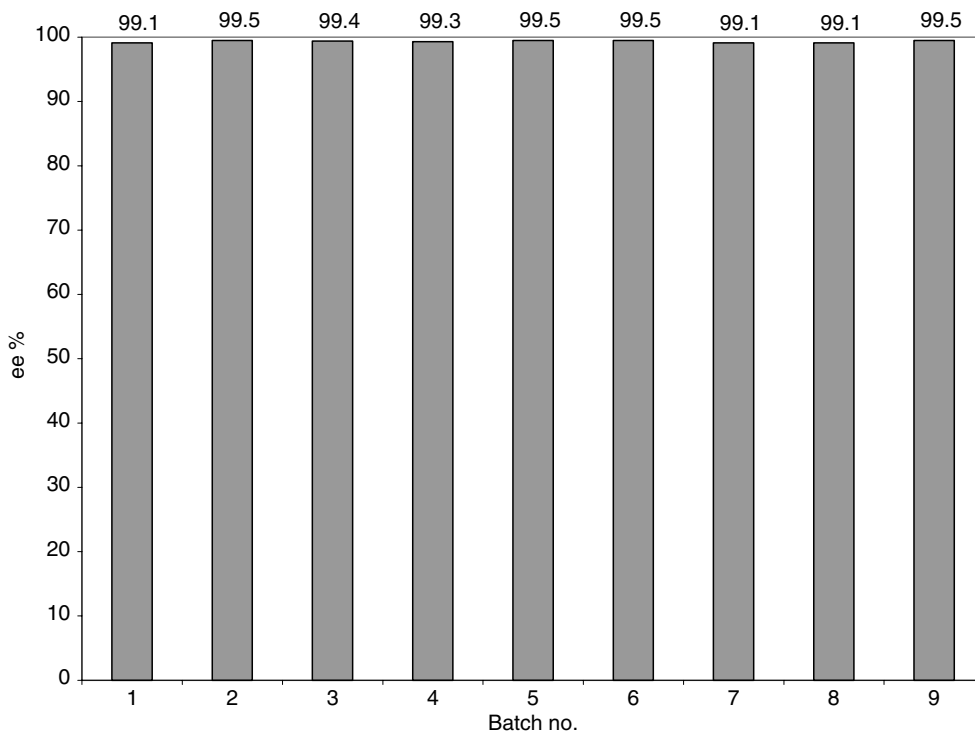


FIGURE 28.1 Long-term stability of PVA-entrapped PaHNL in aqueous media. (From Gröger, H. et al, Bundesforschungsanstalt für Landwirtschaft, Braunschweig, SI 241, 87, 2002.)

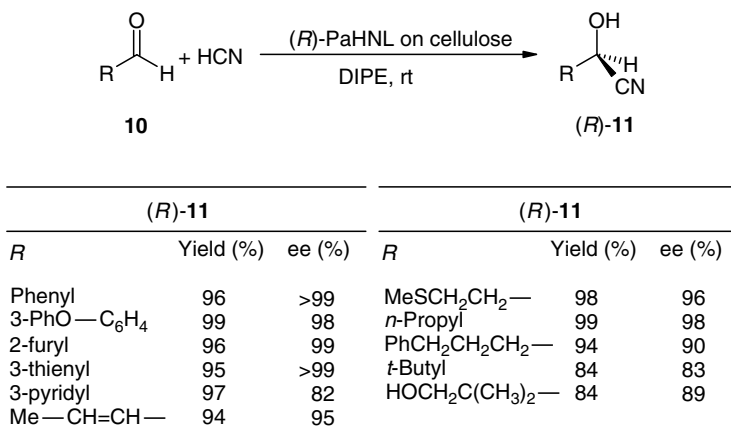
is the encapsulation of the enzymes in polyvinyl alcohol (PVA) [36–38]. The lens-shaped HNL-containing catalyst shows a well-defined particle of diameter 3 to 5 mm and thickness 0.3 to 0.4 mm. PVA-entrapped (*R*)-PaHNL can be applied in buffered aqueous media (pH 3.75) or in a biphasic system [36–38]. The catalyst can be reused without any loss of enantioselectivity (Figure 28.1) [36–38]. (*R*)-PaHNL has also been immobilized by cross-linking enzyme aggregates (CLEA) using glutaraldehyde [38]. The CLEA of (*R*)-PaHNL are stable and can be recycled many times [38].

Recently, the use of ionic liquids instead of organic solvents was published for the biphasic system [39]. For PaHNL and HbHNL, the reaction rates were increased in comparison to organic solvents, while the enantioselectivity was identical [39].

28.3.2 (*R*)-HNL CATALYSIS

(*R*)-PaHNL from almonds (*Prunus amygdalus*), the first enzyme ever used in asymmetric synthesis [40], is not only easily available in large amounts from almonds but is also surprisingly enantioselective when accepting a broad spectrum of substrates (Scheme 28.4) [1–12]. (*R*)-HNLs isolated from other plants do not show any improvements as catalysts for the preparations of (*R*)-cyanohydrins, and therefore cannot compete with (*R*)-PaHNL for applications [41–43].

Recently, cloning of the gene of HNL isoenzyme 5 (PaHNL5) and overexpression in the methylotrophic yeast *Pichia pastoris* was reported [44,45]. The authors stated that the recombinant PaHNL5 shows considerably higher reactivity than PaHNL for reactions of nonnatural substrates like 2-chlorobenzaldehyde [44,45]. However, comparable results (89% yield, 91% ee) can be obtained by working with wild-type enzyme adsorbed on cellulose



SCHEME 28.4 (*R*)-Cyanohydrins (*R*)-**11** by PaHNL-catalyzed addition of HCN to Aldehydes **10** (From Effenberger, F., *Setoselective Biocatalysis*, Marcel Dekker, New York, 2000, P. 321.)

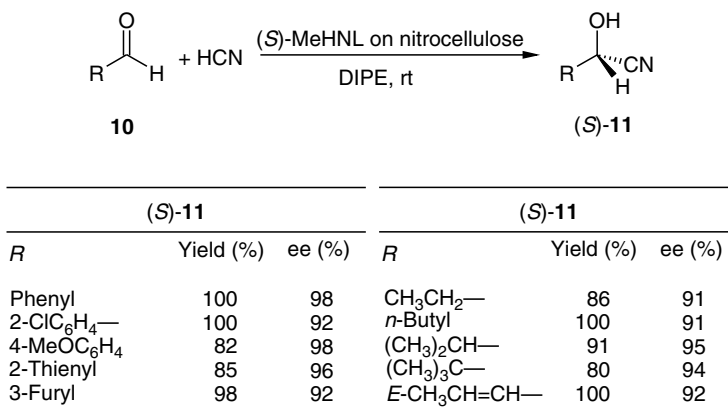
in DIPE as solvent [46]. Even almond powder in a biphasic system (Na-citrate buffer pH 3.3/methyl-*tert*-butylether) gives (*R*)-2-chlorobenzaldehyde cyanohydrin in 94% yield with 83% ee [46,47]. The optimized reaction conditions with almond powder in a two-phase system were successfully applied to the preparation of a large number of (*R*)-cyanohydrins [48,49]. An interesting synthetic variation called “micro-aqueous conditions” was developed by Han et al. by working with almond meal in an organic solvent [50]. This method can be applied in a continuous-flow system [51]. Under optimized flow rates, both aromatic and heteroaromatic aldehydes [51] could be converted to cyanohydrins with 84 to 100% ee. All two-phase reactions described here were optimized empirically. Straathof has developed a mathematical model, which demonstrates that the reaction rate will be influenced by the substrate concentration, by the initial enzyme concentration, and by the volume-specific interfacial area [52].

28.3.3 (*S*)-HNL CATALYSIS

The first (*S*)-HNL used in the preparation of (*S*)-cyanohydrins was isolated from *Sorghum bicolor* (SbHNL). SbHNL was applied in organic [53] as well as in aqueous [54] solution. Although very high ee result with SbHNL as catalyst, this enzyme has two disadvantages. First, only aromatic and heteroaromatic aldehydes are accepted as substrates and second, the availability from natural sources is far more limited than the availability of PaHNL from almonds. Until now, SbHNL could not be obtained in recombinant form.

Two other (*S*)-HNLs, however, which do not show these disadvantages have been developed in the last few years and are now widely used for the synthesis of (*S*)-cyanohydrins. The first is (*S*)-MeHNL, which is isolated from *Manihot esculenta* [55], cloned and overexpressed in *Escherichia coli* [56]; the second is (*S*)-HbHNL from *Hevea brasiliensis*, overexpressed in the yeast *P. pastoris* [57,58]. Since, MeHNL is highly homologous to HbHNL, both enzymes show similar catalytic behavior. Like (*R*)-PaHNL from almonds, (*S*)-MeHNL and (*S*)-HbHNL, respectively, accept a wide range of aldehydes (aromatic, heteroaromatic, aliphatic, and saturated as well as unsaturated) and even ketones as substrates (Scheme 28.5) [10].

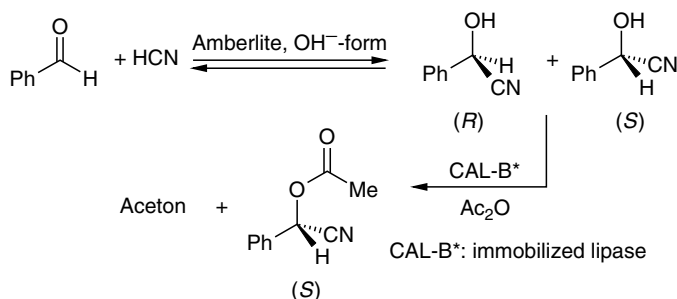
During the last years, the overexpression of both enzymes, MeHNL and HbHNL, has been improved considerably [59], so that large quantities are now cheaply available. Jülich Fine Chemicals, GmbH, Germany, for example, is selling (*S*)-MeHNL in giga units [59].



SCHEME 28.5 (*S*)-Cyanohydrins(*S*)-**10** by MeHNL-catalyzed addition of HCN to aldehydes **11** (From Effenberger, F., *Stereoselective Biocatalysis*, Marcel Dekker, New York, 2000, p. 321.)

28.3.4 LIPASE-CATALYZED PREPARATION OF CHIRAL CYANOHYDRINS

The kinetic resolution of racemic cyanohydrins and cyanohydrin derivatives, respectively by using lipases, has been summarized comprehensively [5,13]. Disadvantages of this method lie not only in the maximum yield of 50%, but also in the racemization tendency of unprotected chiral cyanohydrins under the conditions (pH ≥ 6) used for lipase-catalyzed reactions. This problem was avoided in the synthesis of (1*R*,*cis*, α *S*)-cypermethrine through lipase-catalyzed kinetic resolution of racemic *m*-phenoxybenzaldehyde cyanohydrin acetate by direct acylation of the obtained (*S*)-*m*-phenoxybenzaldehyde cyanohydrin with the corresponding cyclopropane carboxylic acid chloride [60]. The unreacted (*R*)-cyanohydrin is removed and racemized with triethylamine to give the racemic cyanohydrin acetate again, which is returned into the process. The total yield of the optically pure pyrethroide (1*R*,*cis*, α *S*)-cypermethrine referred to the racemic cyanohydrin acetate is more than 80% [60]. The kinetic resolution of racemic cyanohydrin acetates and protection of the formed (*S*)-cyanohydrins was performed with several racemic aromatic cyanohydrin acetates. In this case, both enantiomers are obtained in high optical purity [61]. Recently, the lipase-catalyzed resolution of protected hydroxycyanohydrins was published by Gotor et al. [62]. The dynamic kinetic resolution using lipase has been successfully developed as a new methodology for the preparation of only one enantiomer, starting from racemic cyanohydrins or cyanohydrin derivatives [63,64,65,66]. When choosing optimized experimental conditions, the preparation of only one enantiomer in almost quantitative yield is possible starting from racemic cyanohydrins [63,64,65,66]. (Scheme 28.6)



SCHEME 28.6 Chiral cyanohydrins by dynamic kinetic resolution of racemic cyanohydrins (From Li, Y-K., Stratthof, A.J., and Hanefeld, U., *Tetrahedron Asymmetry*, 13, 739, 2002; Veum, L. and Hanefeld, U., *Tetrahedron Asymmetry*, 15, 3707, 2004.)

28.3.5 SUMMARY OF ENZYME-CATALYZED PREPARATIONS OF CHIRAL CYANOHYDRINS

The HNL-catalyzed addition of HCN to aldehydes is still the single most important method for the preparation of nonracemic cyanohydrins. The usefulness of these enzymes has increased substantially since (*R*)-PaHNL from almonds and two recombinant (*S*)-HNLs (MeHNL and SbHNL) are easily available in large amounts. Many (*R*)- as well as (*S*)-cyanohydrins are manufactured nowadays as intermediates for the production of pharmaceuticals and plant protecting compounds, respectively (see Chapter 5). Further improvements of the dynamic kinetic resolution using lipases could be an enzymatic alternative to HNLs in cases where the starting carbonyl compounds (aldehydes or ketones) are not accepted as substrates by HNLs.

28.4 CRYSTAL STRUCTURES OF HNLs AND MECHANISM OF CYANOGENESIS

Several structurally different types of HNLs occur in nature, which likely originated by convergent evolution from different ancestral proteins [67]. The properties and characteristics of the five most important HNLs for applications in the preparation of chiral cyanohydrins are listed in Table 28.1 [10].

The crystal structures of these five HNLs have only been determined during the last decade, which allowed researchers to establish the mechanistic pathways of cyanogenesis.

28.4.1 CRYSTAL STRUCTURES OF HNLs

A summary of 3D structures of HNLs known so far was recently published by Kratky et al. [68]. The enzyme from almonds (PaHNL) was already crystallized in 1994, and the structure was solved by multiple wavelength anomalous dispersion of a mercury derivative [69]. The first 3D-structure analysis of PaHNL to a crystallographic resolution of 1.5 Å was performed in 2001 [70]. (*R*)-PaHNL from almonds uses FAD as cofactor and is related to oxidoreductases [71,72]. It is interesting to note that the enzyme exhibits HNL activity only in the oxidized form of the cofactor FAD [73]. From indirect evidence, it was inferred that the active site of PaHNL is located close to the isoalloxazine of the cofactor [68].

TABLE 28.1
Properties and Characteristics of HNLs

Enzyme Source	Natural Substrate (Cyanogenic Glycoside)	<i>R/S</i> -Selectivity	Molecular Weight (kDa)	
			Native	Subunit
<i>Prunus amygdalus</i> (Rosacea)	(<i>R</i>)-Mandelonitrile (amygdalin, prunasin)	<i>R</i>	55–80	55–80
<i>Sorghum bicolor</i> (Gramineae)	(<i>S</i>)- <i>p</i> -Hydroxy- mandelonitrile (dhurrin)	<i>S</i>	105	30 and 22
<i>Manihot esculenta</i> (Euphorbiaceae)	Acetone cyanohydrin (linamarin, lotaustralin)	<i>S</i>	92–124	28–30
<i>Hevea brasiliensis</i> (Euphobiaceae)	Acetone cyanohydrin (linamarin)	<i>S</i>	58	30
<i>Linum usitatissimum</i> (Linaceae)	Acetone cyanohydrin (linamarin)	<i>R</i>	82	42

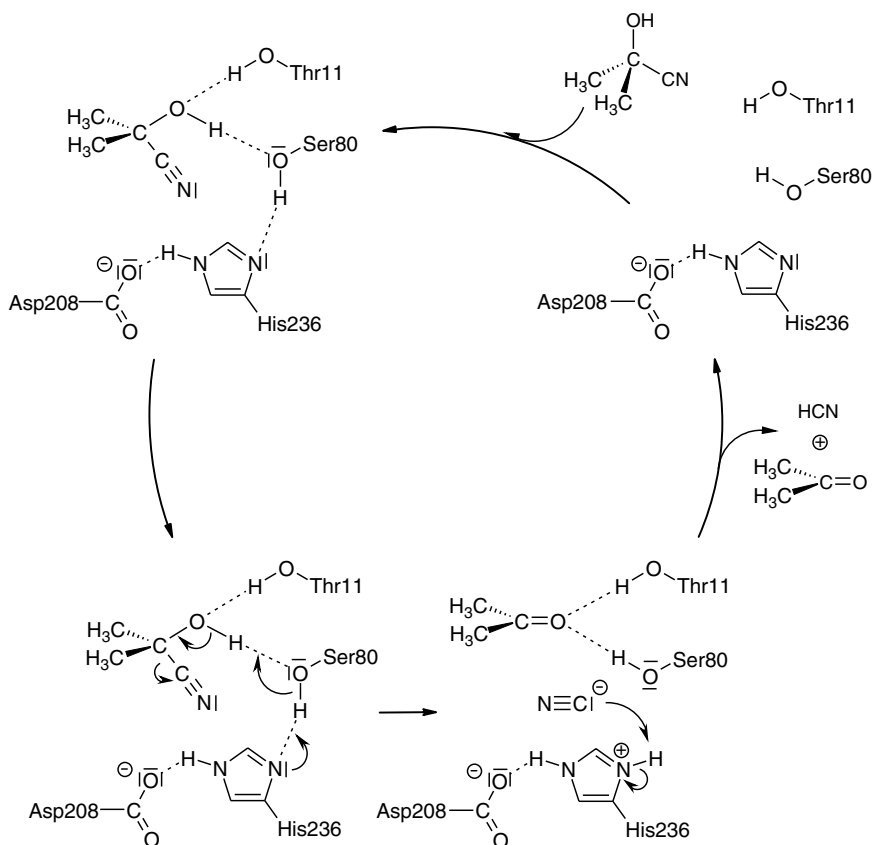
The crystal structure of the HNL from *S. bicolor* was determined in a complex with the inhibitor benzoic acid [74]. The folding pattern of SbHNL is similar to that of the closely related wheat serine carboxy peptidase (CPD-WII) [75] and alcohol dehydrogenase [76]; however, a unique two-amino acid deletion in SbHNL is forcing the putative active-site residues away from the hydrolase binding site toward a small hydrophobic cleft, thereby defining a completely different SbHNL active-site architecture where the classic triad of a carboxy peptidase is missing.

The most extensively investigated HNL structures are those of the highly homologous (76% sequence identity) enzymes from *H. brasiliensis* (HbHNL) and *M. esculenta* (MeHNL), both belonging to the family of α,β -hydrolases [77,78]. The 3D structure of HbHNL was first published in 1996 [79], and an ultrahigh resolution was reported in 1999 [80]. For MeHNL, the crystal structures of the wild-type enzyme complexed with acetone and chloroacetone, respectively, have been reported in 2001 [81,82]. The 3D structures of both enzymes clearly confirm that they belong to the family of α,β -hydrolases. The active site of both enzymes is located inside the protein and is connected to the outside through a small channel, which is covered by a bulky amino acid (tryptophane 128) [81,82]. It was possible to obtain the crystal structure of the complex with the natural substrate acetonecyanohydrin with the active-site mutant Ser80Al of MeHNL [81,82]. This complex allowed determination of the mode of substrate binding in the active site of the enzyme [81,82].

28.4.2 REACTION MECHANISM OF CYANOGENESIS

Although mechanisms for cyanogenesis have been published based on the crystal structure investigations for HNL from *S. bicolor* [74] as well as for PaHNL from almonds [83], here, we will only discuss the thoroughly proven mechanism of the closely related enzymes HbHNL and MeHNL. Originally, a mechanism involving a general acid/base catalysis was proposed for MeHNL on the basis of sequence and mutational analysis [84]. The first suggested mechanism for HbHNL, based on its crystal structure, was a covalently bound reaction intermediate in analogy to the accepted mechanism for hydrolases [79]. Later, this possibility was dismissed on the basis of structural data of HbHNL-inhibitor complexes [85]. Combining x-ray crystallography and site-directed mutagenesis results in a mechanism of cyanogenesis in which His236 acts as a general base and abstracts a proton from Ser80, thereby allowing proton transfer from the hydroxy group of acetonecyanohydrin to Ser80. The His236 imidazolium cation then facilitates the leaving of the nitrile group by proton transfer (Scheme 28.7) [81,82]. In a recent publication [86], it was demonstrated that the active-site lysine residue (Lys237 in MeHNL and Lys236 in HbHNL) is also necessary for the catalytic activity of these enzymes. The structure of selected active site residues complexed with acetonecyanohydrin is depicted in Figure 28.2 to explain the stereoselectivity.

The active site of MeHNL is located in a cavity buried inside the enzyme and is only accessible through a narrow tunnel that is covered by the large amino acid Trp128. As shown, the ketone carbonyl is hydrogen bonded to Ser80 and Thr11. One methyl group of acetone (labeled C1) is held in position by van der Waals contacts to Leu149, Thr11, and Ile12. The side chains of these three residues define a small hydrophobic site S_1 . The second methyl group (labeled C3) points in the opposite direction toward the active-site tunnel, defining the putative second subsite, S_2 in the binding cavity. Asymmetrical carbonyl compounds, for instance aldehydes, are fixed in a way that the smaller substituent (hydrogen) is situated in S_1 and the larger group (R) in S_2 . This binding mode suggests that the incoming cyanide exclusively attacks the *Si*-face of the carbonyl compound when bound to the active site of the enzyme. Thus implies that HCN has to be deprotonated by His236, which is located on the *Si*-face of the carbonyl compound to be consistent with the (*S*)-stereospecificity of the addition [81,82].



SCHEME 28.7 Catalytic mechanism of cyanogenesis by MeHNL.

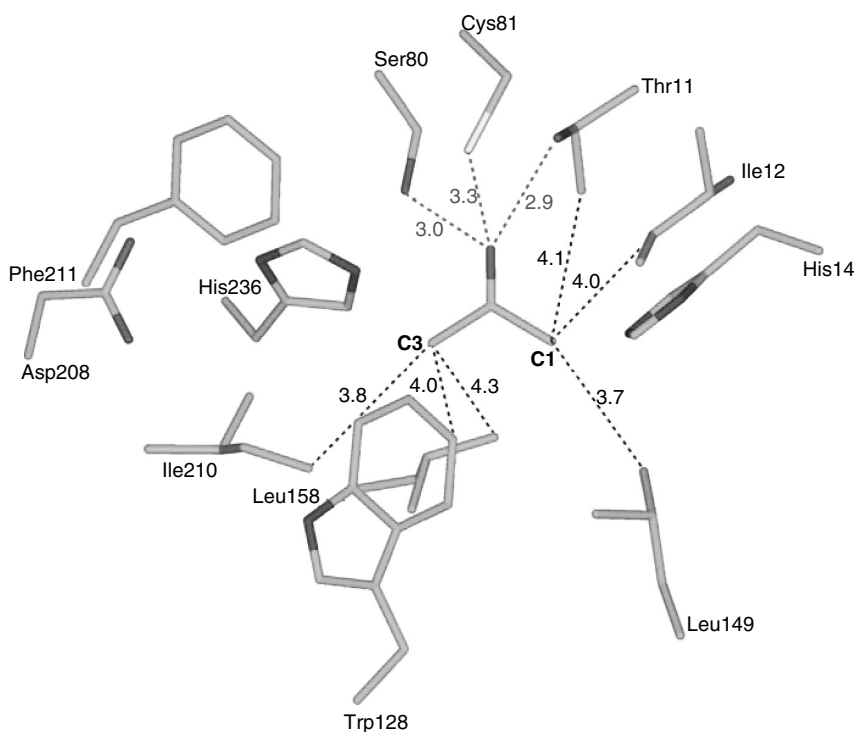


FIGURE 28.2 (See color insert following page 526) Structure of selected active-site residues of hydroxynitrile lyase from *Manihot esculenta* (MeHNL) complexed with acetone.

28.4.3 CHANGING SUBSTRATE SPECIFICITY AND STEREOSELECTIVITY BY APPLYING MUTANTS OF MEHNL

Although MeHNL accepts a wide range of carbonyl compounds as substrates [9–11], the catalytic activity for bulky substrates is limited with respect to conversion and ee, as demonstrated for the preparation of (*S*)-3-phenoxybenzaldehyde cyanohydrin, an important starting compound for pyrethroids [9–11]. As mentioned earlier, the x-ray crystal structure of MeHNL has revealed that the active site of the enzyme is accessible by a narrow channel, where the channel entrance is capped by tryptophan (Trp128). Substitution of Trp128 by amino acids with decreasing size (alanine, cysteine, leucine, and tyrosine) gives the corresponding wt-MeHNL mutants. MeHNL-W128A, for example, could be prepared and overexpressed [87]. It is obvious from the crystal structure of MeHNL-W128A that the entrance to the active site of the enzyme is significantly less hindered, which facilitates access of sterically demanding substrates to the active site. Comparable reactions of 3-phenoxybenzaldehyde with wt-MeHNL and MeHNL-W128A in both aqueous citrate buffer and biphasic system of water/MTBE reveal the superiority of the W128A mutant. The aldehyde was converted quantitatively with high ee using the mutant W128A, nearly independent of the amount of enzyme present, with a reaction rate of 57 g/L/h [87]. Channel mutants differ not only in rates and ee-values, but can even invert the configuration of the cyanohydrins prepared [88]. In the MeHNL-catalyzed addition of HCN to *rac*-2-phenylpropionaldehyde, the (*R*)-enantiomer reacts with wt-MeHNL and with all MeHNL Trp128 mutants highly (*S*)-selective to give exclusively the (2*S*,3*R*)-diastereoisomers. The (*S*)-enantiomer of *rac*-3-phenylpropionaldehyde, however, reacts (*S*)-selectively only with wt-MeHNL to give the (2*S*,3*S*)-diastereoisomer, while the MeHNL-W128A mutant is exclusively (*R*)-selective resulting in the (2*R*,3*S*)-diastereoisomer [88]. The inversion of stereoselectivity can be explained and rationalized through crystal structure-based molecular modeling [88].

28.4.4 SUMMARY OF CRYSTAL STRUCTURES OF HNLs AND MECHANISM OF CYANOGENESIS

Since HNLs have originated by convergent evolution from different ancestral proteins, the 3D structure of the various HNLs differ considerably. In all cases, however, a general base catalysis is responsible for the catalytic activity of HNLs. During the last decade, the 3D structures of the most important HNLs (PaHNL, HbHNL, MeHNL, LuHNL, and SbHNL) have been determined. It is possible to formulate mechanisms for cyanogenesis by combining these structural informations with kinetics of certain mutants of the active site of HNLs. During the last 5 y, the mechanisms of cyanogenesis for both MeHNL and HbHNL was unambiguously elucidated.

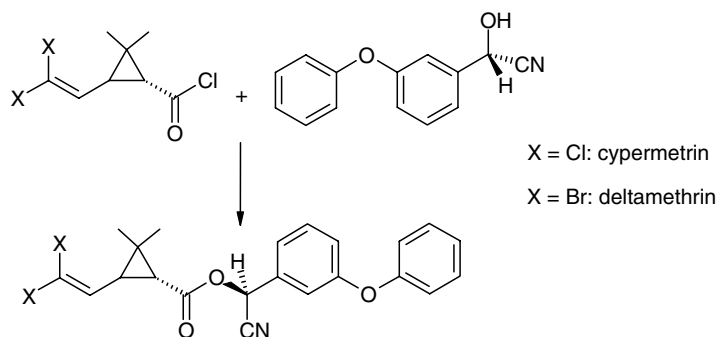
28.5 IMPORTANT APPLICATIONS OF HNLs IN ORGANIC SYNTHESIS

The fundamentals of HNL-catalyzed syntheses of nonracemic cyanohydrins have been well-established [3–12]. Some applications of HNLs in organic synthesis are summarized in several recent review articles [89–92]. Most of the possible stereoselective follow-up reactions of chiral cyanohydrins have also been conceptually investigated [3–12]. Therefore, the application of chiral cyanohydrins will play a major role in the preparation of biologically active compounds with stereogenic centers, for the synthesis of pharmaceuticals and agrochemicals. For technical applications, the enzymes used must be available in sufficient amounts and reasonably priced. For the preparation of (*R*)-cyanohydrins, the wild-type PaHNL from almond fulfills both requirements. For the synthesis of (*S*)-cyanohydrins, the recombinant HNLs from rubber tree

(HbHNL) [93,94] and cassava (MeHNL) [95] have been optimized to high efficiencies. They are produced in giga units [95].

28.5.1 BIOLOGICALLY ACTIVE CYANOHYDRIN ESTERS

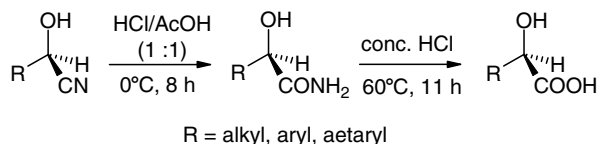
The insecticidal activities of cyanohydrin esters are well known and investigated [96]. The esters of chiral cyanohydrins with chiral pyrethrum acids are the most important insecticides [93]. The majority of the commercially important pyrethroids contain (*S*)-3-phenoxybenzaldehyde cyanohydrin as alcohol component (Scheme 28.8). Nowadays, the technical production of this (*S*)-cyanohydrin is performed almost exclusively through HNL-catalyzed addition of HCN to 3-phenoxybenzaldehyde using HbHNL or MeHNL, as catalysts. As mentioned earlier (Chapter 4.3), the channel mutant MeHNL-W128A is especially advantageous for the reaction of sterically demanding substrates like 3-phenoxybenzaldehyde [87].



SCHEME 28.8 Pyrethroids containing cyanohydrin moieties.

28.5.2 CHIRAL 2-HYDROXY ACIDS

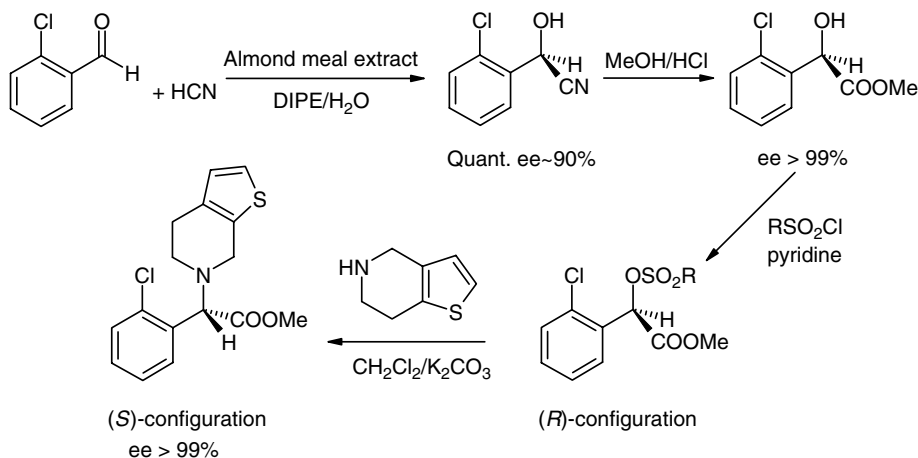
In contrast to the abundant 2-amino acids, only a few nonracemic 2-hydroxy acids are found in nature. They have considerable synthetic promise [97]. Hydrolysis of chiral cyanohydrins offers a general route to (*R*)- as well as (*S*)-2-hydroxy carboxylic acids. (*R*)- and (*S*)-Cyanohydrins are easily hydrolyzed in concentrated hydrochloric acid to give the corresponding (*R*)- and (*S*)-2-hydroxy acids, respectively, in excellent yields and with complete retention of configuration (Scheme 28.9) [5]. Under mild conditions (e.g., low temperature and short reaction times), the carboxylic acid amides can be obtained selectively (Scheme 28.9) [98].



SCHEME 28.9 Selective hydrolysis of (*R*)-cyanohydrins under complete retention of configuration.

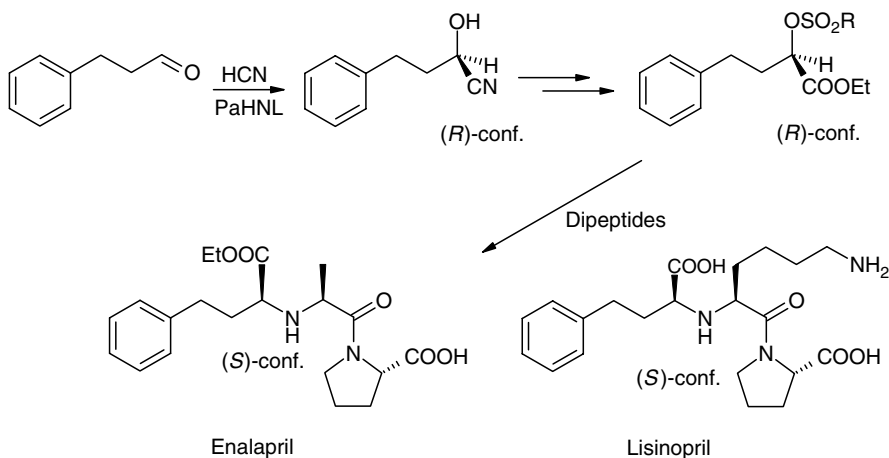
Aromatic α -hydroxy carboxylic acids are of special interest [35]. Among them, enantiomerically pure mandelic acid and substituted derivatives thereof are regarded as the most important representatives from a commercial point of view [35]. The great synthetic potential of optically active α -hydroxy acids lies in the possibility to activate the hydroxyl function, for example, by sulfonylation [99–101]. The activated derivatives react readily with any kind of nucleophile under complete inversion of configuration [99–101].

The production of the blockbuster Clopidogrel (Plavix) is a convincing example for the synthetic potential of follow-up reactions of optically active cyanohydrins [102]. In the first step, (*R*)-2-chlorobenzaldehyde cyanohydrin is prepared by almond meal catalyzed by the addition of HCN to 2-chlorobenzaldehyde. The cyanohydrin is then transformed into the corresponding α -hydroxy carboxylic ester, which can be reacted with tetrahydrothieno[3,2-*c*]pyridine after activation with phenyl sulfonylchloride to give clopidogrel (Scheme 28.10).



SCHEME 28.10 Stereoselective synthesis of clopidogrel (From Bousquet, A. and Musolino, A., PCT Int. Appl. 1999, Wo 9918110 A1, CAN 130: 296510, 1999.)

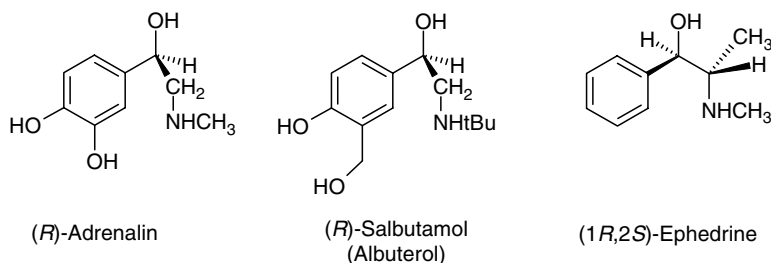
Another class of pharmaceuticals that can be synthesized through optically active cyanohydrins are angiotensin-converting enzyme (ACE) inhibitors [103]. (*R*)-3-Phenylpropionaldehyde cyanohydrin is an important starting cyanohydrin for the preparation of ACE inhibitors. After transformation of the cyanohydrin into the corresponding carboxylic ester and activation of the hydroxyl function by sulfonylation, the reaction with dipeptides, for example, yields under inversion of configuration stereoselectively ACE inhibitors known as enalapril and lisinopril (Scheme 28.11) [103,104].



SCHEME 28.11 Stereoselective synthesis of ACE-inhibitors.

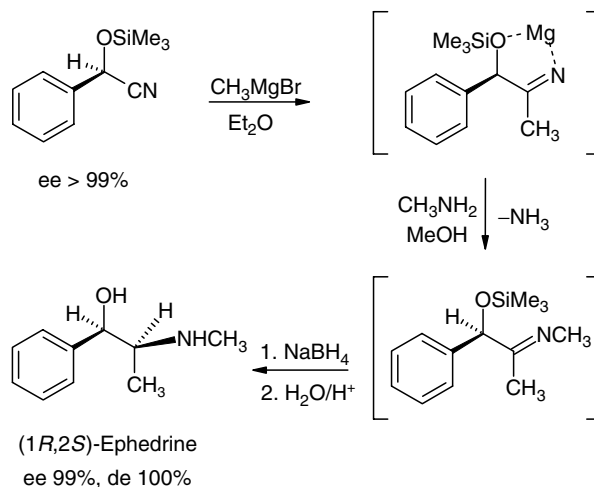
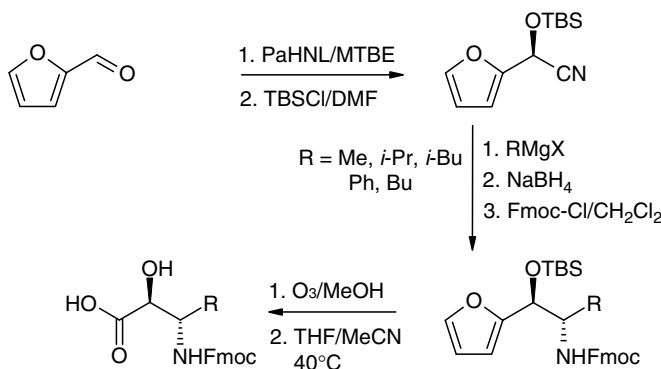
28.5.3 NONRACEMIC 1,2-AMINO ALCOHOLS

1,2-Amino alcohols have a broad spectrum of biological activity [105]. They can be categorized as adrenalin-like with one chiral center at C-1 or as ephedrine-like with two chiral centers at C-1 and C-2 (Scheme 28.12). Although it is known that only the compounds with (1*R*)- and (1*R*,2*S*)-configuration, respectively, are responsible for the desired biological activity, in most cases racemates are still applied as pharmaceuticals. Since some of the other enantiomers seem to have undesirable side effects [106–109], stereoselective syntheses of 1,2-amino alcohols are becoming increasingly important.



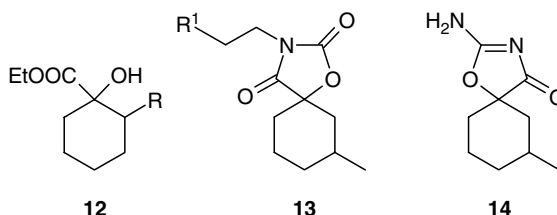
SCHEME 28.12 Biologically active 1,2-amino alcohols.

A variety of methods have been developed for the stereoselective preparation of 1,2-amino alcohols [106–109]. However, the stereoselective preparation of amino alcohols starting from chiral cyanohydrins is in many cases easier and more efficient than other methods. Both free and *O*-protected chiral cyanohydrins can be hydrogenated with LiAlH_4 without any racemization to give adrenalin-type 1,2-amino alcohols. The use of *tert*-butyldimethylsilyl(TBDMS)-protecting groups allows partial hydrogenation with Diisobutylaluminum hydride (DIBALH) and transformation of the formed imino intermediates, for example: exchanging =NH against =NR. Subsequent hydrogenation gives the pharmacologically important *N*-alkyl-substituted (1*R*)-2-amino alcohols (Scheme 28.12) [110,111]. Ephedrine-like 2-amino alcohols can be prepared stereoselectively from *O*-protected chiral cyanohydrins by the addition of a Grignard reagent and by subsequent hydrogenation with NaBH_4 (Scheme 28.13) [112,113]. Again, NH_3 in the imino intermediate can be exchanged by primary amines to get after hydrogenation the *N*-substituted 1,2-amino alcohols. The hydrogenation of the imino intermediates is highly diastereoselective due to a chelate-controlled reaction; *erythro* products are formed almost exclusively [113]. (1*R*,2*S*)-Ephedrine is commercially produced by a fermentation process that is usually limited to specific substrates. In contrast, almost any structural variation of the 1,2-amino alcohol is possible when starting from chiral cyanohydrins. The stereoselective preparation of heteroatomic analogs of *L*-ephedrine is an interesting example of this synthetic methodology [114]. In the case of furyl-1,2-amino alcohols, it is possible to transform the furyl group into a carboxyl group by ozonization, which opens up the possibility of a stereoselective route to 2-hydroxy-3-amino carboxylic acids (Scheme 28.14) [115]. Another synthetic possibility is the addition of allyl Grignard to the nitrile group. Ozonolysis of the acetylated amino alcohols affords the corresponding aldehydes, which can be oxidized to give the optically active β -hydroxy- α -amino acids [49].

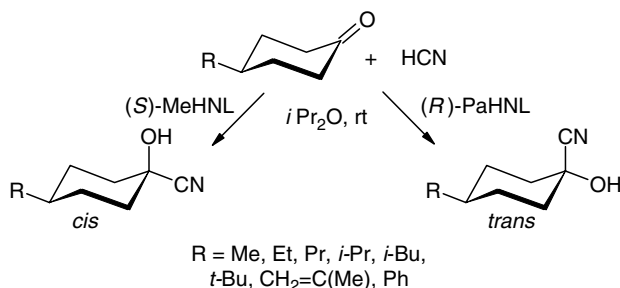
**SCHEME 28.13** Stereoselective synthesis of R(2S)-Ephedrine.**SCHEME 28.14** Synthesis of (3S)-2-hydroxy-3-amino acids (From Tromp, R.A., et al., *Tetrahedron Asymmetry*, 14, 1645, 2003.)

28.5.4 CYANOHYDRINS OF SUBSTITUTED CYCLOHEXANONES

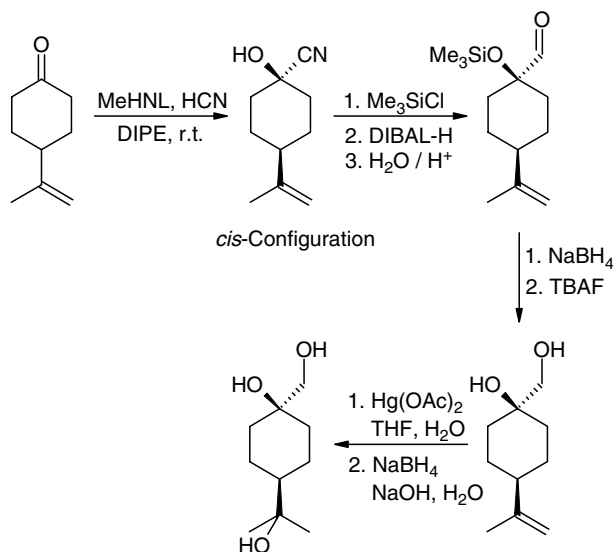
Monosubstituted cyclohexanone cyanohydrins, which can easily be hydrolyzed to the corresponding α -hydroxy carboxylic acids, are important as both pharmaceuticals and plant protective agents [116–118]. This structural unit is also common in several natural products [119–122]. Some examples are given in Scheme 28.15. Although each of the compounds **12**, **13**, and **14** has two stereogenic centers, stereoselective syntheses of these compounds have been published only recently [123].

**SCHEME 28.15** Examples of substituted hydroxy cyclohexanone carboxylic acids.

The HNL-catalyzed addition of HCN to 4-substituted cyclohexanones, which do not contain a prochiral center, is very interesting and completely unexpected [124,125]. Cyclohexanones are excellent substrates for both PaHNL and MeHNL, respectively, which surprisingly exhibit high *cis/trans*-selectivity. The (*R*)-PaHNL-catalyzed reaction affords almost exclusively the *trans*-isomers, while with (*S*)-MeHNL the *cis*-addition is favored (Scheme 28.16) [124,125]. Since the chemical addition of HCN to 4-substituted cyclohexanones always results in mixtures of *cis/trans*-isomers, a stereoselective addition is of great importance. For example, in the chemical addition of HCN to 4-alkyl substituted cyclohexanones, the *cis/trans*-ratio is ~13:87. Important natural products that can be readily obtained from corresponding cyclohexanone cyanohydrins; however, have exclusively *cis*-configuration [119–122]. Therefore, the chemical addition of HCN would be highly unfavorable because of the large *trans*-ratio. However, the *cis/trans*-ratio in many cases is as high as 98:2 with MeHNL as catalyst [124,125]. The synthesis of the natural monoterpenes *cis-p*-menth-8-ene-1,7-diol and *cis-p*-menthane-1,7,8-triol by this new methodology is summarized in Scheme 28.17 [120].



SCHEME 28.16 *Cis/trans*-selectivity of HNL-catalyzed additions of HCN to 4-substituted cyclohexanones (From Effenberger, F., Kobler, C., and Roos, J., *Angew. Chem. Int. Ed.*, 41, 1876, 2002; Effenberger, F., et al., *Can. J. Chem.*, 80, 671, 2002.)



SCHEME 28.17 Synthesis of biologically active monoterpenes (From Kobler, C. and Effenberger, F., *Chem. Eur. J.*, 11, 2783, 2005.)

28.6 CONCLUSION

The synthetic potential of chiral cyanohydrins for the stereoselective preparation of biologically active compounds (pharmaceuticals and plant protective agents) has been known for more than 10 y [3–12]. The main objective was therefore to summarize the progress achieved during the last 5 y, with special emphasis on improvements in chemical and optical yields of the prepared chiral cyanohydrins, on simplification of the reaction procedures, and on developed conditions for large-scale productions of commercially interesting chiral cyanohydrins. The HNL-catalyzed mechanism of cyanogenesis has been elucidated unambiguously from x-ray structures of the most important HNLs and site-directed mutagenesis. It was possible to improve yields and stereoselectivity in a straightforward manner through knowledge of the active site and the reaction mechanism of the enzymes. Now not only (*R*)-PaHNL from almonds is available in more or less unlimited amounts, but the recombinant (*S*)-HNLs from cassava (MeHNL) and rubber tree (HbHNL) are also produced in giga units, the application of HNLs in large-scale productions of chiral cyanohydrins as intermediates for the stereoselective synthesis of pharmaceuticals and agrochemicals has become possible. Some of the most important technical applications of chiral cyanohydrins have been summarized in [Section 28.5](#).

REFERENCES

1. North, M., Synthesis and applications of non-racemic cyanohydrins and α -amino-nitriles, *Tetrahedron*, 60, 10371, 2004.
2. Effenberger, F., Ziegler, T., and Förster, S., Enzyme-catalyzed cyanohydrin synthesis in organic solvents, *Angew. Chem. Int. Ed. Engl.*, 26, 458, 1987.
3. North, M., Catalytic asymmetric cyanohydrin synthesis, *Synlett*, 807, 1993.
4. North, M., Introduction of the cyano group by addition to a carbonyl group, *Sci. Synth.*, 19, 235, 2004.
5. Effenberger, F., Synthesis and reactions of optically active cyanohydrins, *Angew. Chem. Int. Ed. Engl.*, 33, 1555, 1994.
6. Kanerva, L.T., Biocatalytic ways to optically active 2-amino-1-phenylethanols, *Acta Chem. Scand.*, 50, 234, 1996.
7. Schmidt, M. and Griengl, H., Oxynitrilases: from cyanogenesis to asymmetric synthesis, *Top. Curr. Chem.*, 200, 193, 1999.
8. Gregory, R.J.H., Cyanohydrins in nature and the laboratory: biology, preparations, and synthetic applications, *Chem. Rev.*, 99, 3649, 1999.
9. Effenberger, F., Enzyme-catalyzed preparation and synthetic applications of optically active cyanohydrins, *Chimia*, 53, 3, 1999.
10. Effenberger, F., Hydroxynitrile lyases in stereoselective synthesis, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, p. 321.
11. Effenberger, F., Förster, S., and Wajant, H., Hydroxynitrile lyases in stereoselective catalysis, *Curr. Opin. Biotechnol.*, 11, 532, 2000.
12. Brussee, J. and van der Gen, A., Biocatalysis in the enantioselective formation of chiral cyanohydrins, valuable building blocks in organic synthesis, in *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, pp. 289–320.
13. North, M., Synthesis and application of non-racemic cyanohydrins, *Tetrahedron Asymmetry*, 14, 147, 2003.
14. Brunel, J.-M. and Holmes, I.P., Chemically catalyzed asymmetric cyanohydrin syntheses, *Angew. Chem. Int. Ed. Engl.*, 43, 2752, 2004.
15. Corey, E.J. and Wang, Z., Enantioselective conversion of aldehydes to cyanohydrins by a catalytic system with separate chiral binding sites for aldehydes and cyanide components, *Tetrahedron Lett.*, 34, 4001, 1993.

16. Ryu, D.H. and Corey, E.J., Enantioselective cyanosilylation of ketones catalyzed by a chiral oxazaborolidiniumion, *J. Am. Chem. Soc.*, 127, 5384, 2005.
17. Brunel, J.-M., Legrand, O., and Buono, G., Enantioselective trimethylsilyl cyanation of aromatic aldehydes catalyzed by titanium alkoxide-chiral-*o*-hydroxyarylphosphine oxides, *Tetrahedron Asymmetry*, 10, 1979, 1999.
18. Hamashima, Y., et. al., Highly enantioselective cyanosilylation of aldehydes catalyzed by a Lewis-acid–Lewis-base bifunctional catalyst, *Tetrahedron*, 57, 805, 2001.
19. Hamashima, Y., Kanai, M., and Shibasaki, M., Catalytic enantioselective cyanosilylation of ketones, *J. Am. Chem. Soc.*, 122, 7412, 2000.
20. Hamashima, Y., Kanai, M., and Shibasaki, M., Catalytic enantioselective cyanosilylation of ketones: improvement of enantioselectivity and catalyst turn-over by ligand tuning, *Tetrahedron Lett.*, 42, 691, 2001.
21. Yabu, K., et al., Switching enantiofacial selectivities using one chiral source: catalytic enantioselective synthesis of the key intermediate for (20*S*)-camptothecin family by (*S*)-selective cyanosilylation of ketones, *J. Am. Chem. Soc.*, 123, 9908, 2001.
22. Masumoto, S., et al., A practical synthesis of (*S*)-oxybutynin, *Tetrahedron Lett.*, 43, 8647, 2002.
23. Yabu, K., et al., Studies toward practical synthesis of (20*S*)-camptothecin family through catalytic enantioselective cyanosilylation of ketones: improved catalyst efficiency by ligand-tuning, *Tetrahedron Lett.*, 43, 2923, 2002.
24. Belokon, Y., et al., Asymmetric addition of trimethylsilyl cyanide to aldehydes catalyzed by chiral (salen)Ti(IV) complexes, *J. Chem. Soc. Perkin Trans. 1*, 1293, 1997.
25. Tararov, V.I., et al., First structurally defined catalyst for the asymmetric addition of trimethylsilyl cyanide to benzaldehyde, *Chem. Commun.*, 387, 1998.
26. Belokon, Y.N., et al., The asymmetric addition of trimethylsilyl cyanide to aldehydes catalyzed by chiral (salen)titanium complexes, *J. Am. Chem. Soc.*, 121, 3968, 1999.
27. Belokon, Y.N., et al., Catalytic asymmetric synthesis of *O*-acetyl cyanohydrins from KCN; Ac₂O and aldehydes, *Chem. Commun.*, 244, 2002.
28. Belokon, Y.N., et al., Synthetic and mechanistic studies on asymmetric cyanohydrin synthesis using a titanium(salen)bimetallic catalyst, *Tetrahedron*, 60, 10433, 2004.
29. Chen, F.-X., et al., Enantioselective cyanosilylation of ketones by a double-activation method with an aluminium complex and an N-oxide, *Chem. Eur. J.*, 10, 4790, 2004.
30. Holmes, I.P. and Kagan, H.B., The asymmetric addition of trimethylsilylcyanide to aldehydes catalyzed by anionic chiral nucleophiles, Part 2, *Tetrahedron Lett.*, 41, 7457, 2000.
31. Hatano, M., et al., Chiral lithium binaphtholate aqua complex as a highly effective asymmetric catalyst for cyanohydrin synthesis, *J. Am. Chem. Soc.*, 127, 10776, 2005.
32. Li, Y., et al., Highly enantioselective cyanosilylation of aldehydes catalyzed by novel β -amino alcohol–titanium complexes, *J. Org. Chem.*, 69, 7910, 2004.
33. Huang, W., et al., Polymeric salen–Ti(IV) complex catalyzed asymmetric synthesis of *O*-acetylcyanohydrins from KCN, Ac₂O and aldehydes, *Tetrahedron*, 60, 10469, 2004.
34. Breuer, M., et al., Industrial methods for the production of optically active intermediates, *Angew. Chem. Int. Ed. Engl.*, 43, 788, 2004.
35. Gröger, H., Enzymatic routes to enantiomerically pure aromatic α -hydroxy carboxylic acids: a further example for the diversity of biocatalysis, *Adv. Synth. Catal.*, 343, 547, 2001.
36. Gröger, H., et al., Asymmetric synthesis of an (*R*)-cyanohydrin using enzymes entrapped in lens-shaped gels, *Org. Lett.*, 3, 1969, 2001.
37. Gröger, H., et al., Practical aspects of encapsulation technologies, in *Landbauforschung Völkerröde*, Prüae, U. and Vorlop, K.-D., Eds., Bundesforschungsanstalt für Landwirtschaft, Braunschweig, SI 241, 87, 2002.
38. van Langen, L.M., et al., Cross-linked aggregates of (*R*)-oxynitrilase: a stable, recyclable biocatalyst for enantioselective hydrocyanation, *Org. Lett.*, 7, 327, 2005.
39. Gaisberger, R.P., Fechter, M.H., and Griengl, H., The first hydroxynitrile lyase catalyzed cyanohydrin formation in ionic liquids, *Tetrahedron Asymmetry*, 15, 2959, 2004.
40. Rosenthaler, L., Enzyme-effected asymmetric synthesis, *Biochem. Z.*, 14, 238, 1908.

41. Kiljunen, E. and Kanerva, L., Novel (*R*)-oxynitrilase sources for the synthesis of (*R*)-cyanohydrins in diisopropylether, *Tetrahedron Asymmetry*, 8, 1225, 1997.
42. Luna, H., et al., New potential sources of oxynitrilase enzymes, *Rev. Soc. Chim. Mex.*, 41, 111, 1997.
43. Solis, A., et al., New sources of (*R*)-oxynitrilase: capulin (*Prunus capuli*) and Mamey (*Mammea americana*), *Biotechnol. Lett.*, 20, 1183, 1998.
44. Glieder, A., et al., Comprehensive step-by-step engineering of an (*R*)-hydroxynitrile lyase for large-scale asymmetric synthesis, *Angew. Chem. Int. Ed. Engl.*, 42, 4815, 2003.
45. Weis, R., et al., Biocatalytic conversion of unnatural substrates by recombinant almond R-HNL isoenzyme 5, *J. Mol. Cat., B Enzym.*, 29, 211, 2004.
46. Bühler, H., Optimierung der (*S*)-Hydroxynitril lyase aus *Manihot esculenta* durch gezielte Mutationen—Anwendungen von optisch aktiven Cyanhydrinen in der Synthese, Dissertation, Universität Stuttgart, Stuttgart, Germany, 2000.
47. Kirschbaum, B., Wilbert, G., and Effenberger, F., Preparation of optically active cyanohydrins using (*R*)-oxynitrile lyase, U.S. Pat. Appl. Publ. (2002) US 202052523 A1, CAN 136:355075.
48. Effenberger, F. and Gaupp, S., Enzyme-catalyzed reactions. 35. Stereoselective substitution of (*R*)-2-(sulfonyloxy)nitriles with sulfur nucleophiles, *Tetrahedron Asymmetry*, 10, 1765, 1999.
49. Roos, J. and Effenberger, F., Stereoselective synthesis of β -amino- γ -butyrolactones, *Tetrahedron Asymmetry*, 13, 1855, 2002.
50. Han, S., Lin, G., and Li, Z., Synthesis of (*R*)-cyanohydrins by crude (*R*)-oxynitrilase-catalyzed reactions in micro-aqueous medium, *Tetrahedron Asymmetry* 9, 1835, 1998.
51. Chen, P.-R., et al., A practical high throughput continuous process for the synthesis of chiral cyanohydrins, *J. Org. Chem.*, 67, 8251, 2002.
52. Straathof, A.J., Enzymatic catalysis via liquid–liquid interfaces, *J. Biotechnol. Bioeng.*, 83, 371, 2003.
53. Effenberger, F., et al., Enzyme-catalyzed reactions. 5. Enzyme-catalyzed synthesis of (*S*)-cyanohydrins and subsequent hydrolysis to (*S*)- α -hydroxy-carboxylic acids, *Tetrahedron Lett.*, 31, 1249, 1990.
54. Niedermeyer, U. and Kula, M.-R., Enzyme-catalyzed synthesis of (*S*)-cyanohydrins, *Angew. Chem. Int. Ed. Engl.*, 29, 386, 1990.
55. Wajant, H., et al., Acetone cyanohydrin lyase from *Manihot esculenta* (cassava) is serologically distinct from other hydroxynitrile lyases, *Plant Sci.*, 108, 1, 1995.
56. Foerster, S., et al., The first recombinant hydroxynitrile lyase and its application in the synthesis of (*S*)-cyanohydrins, *Angew. Chem. Int. Ed. Engl.*, 35, 437, 1996.
57. Schmidt, M., et al., Preparation of optically active cyanohydrins using the (*S*)-hydroxynitrile lyase from *Hevea brasiliensis*, *Tetrahedron*, 52, 7833, 1996.
58. Hasslacher, M., et al., Molecular cloning of the full-length cDNA of (*S*)-hydroxynitrile lyase from *Hevea brasiliensis*, *J. Biol. Chem.*, 271, 5884, 1996.
59. Effenberger, F., Wajant, H., and Förster, S., Method for the production of hydroxynitrile lyases, PCT Int. Appl. (2001), WO 200148178 A1, CAN 135:45300.
60. Roos, J., Stelzer, U., and Effenberger, F., Enzyme-catalyzed reactions. 34. Synthesis of (1*R*,*cis*, α *S*)-cypermethrine via lipase catalyzed kinetic resolution of racemic *m*-phenoxybenzaldehyde cyanohydrin acetate, *Tetrahedron Asymmetry*, 9, 1043, 1998.
61. Veum, L., et al., Enantioselective synthesis of protected cyanohydrins, *Eur. J. Org. Chem.*, 1516, 2002.
62. de Gonzalo, G., et al., Enzymatic acylation reactions on ω -hydroxycyanohydrins, *Tetrahedron*, 60, 10525, 2004.
63. Paizs, C., et al., Candida antarctica lipase A in the dynamic resolution of novel furylbenzothiazole-based cyanohydrin, *Tetrahedron Asymmetry*, 14, 619, 2003.
64. Paizs, C., Biocatalytic enantioselective preparation of phenothiazine-based cyanohydrin acetates: kinetic and dynamic kinetic resolution, *Tetrahedron*, 60, 10533, 2004.
65. Li, Y.-X., Stratthof, A.J., and Hanefeld, U., Enantioselective formation of mandelonitrile acetate: investigation of a dynamic kinetic resolution, *Tetrahedron Asymmetry*, 13, 739, 2002.

66. Veum, L. and Hanefeld, U., Enantioselective formation of mandelonitrile acetate: investigation of a dynamic kinetic resolution II, *Tetrahedron Asymmetry*, 15, 3707, 2004.
67. Wajant, H. and Effenberger, F., Hydroxynitrile lyases of higher plants, *Biol. Chem. Hoppe-Seyler*, 377, 611, 1996.
68. Gruber, K. and Kratky, C.J., Biopolymers for biocatalysis: structure and catalytic mechanism of hydroxynitrile lyases, *J. Polym. Sci. [A]*, 42, 479, 2004.
69. Lauble, H., et al., Crystallization and preliminary x-ray diffraction studies of mandelonitrile lyase of almonds, *Proteins Struct. Funct. Genet.*, 19, 343, 1994.
70. Dreveny, I., et al., The hydroxynitrile lyase from almond: a lyase that looks like an oxidoreductase, *Structure*, 9, 803, 2001.
71. Cavener, D.R., GMC oxidoreductases. A newly defined family of homologous proteins with diverse catalytic activities, *J. Mol. Biol.*, 223, 811, 1992.
72. Kiess, M., Hecht, H.J., and Kalisz, H.M., Glucose oxidase from *Penicillium amagasakiense*. Primary structure and comparison with other glucose-methanol-choline (GMC) oxidoreductase, *Eur. J. Biochem.*, 252, 90, 1998.
73. Baerwald, K.R. and Jaenicke, L., D-Hydroxynitrile lyase: involvement of the prosthetic flavin adenine dinucleotide in enzyme activity, *FEBS Lett.*, 90, 255, 1978.
74. Lauble, H., et al., Crystal structure of hydroxynitrile lyase from *Sorghum bicolor* in complex with the inhibitor benzoic acid: a novel cyanogenic enzyme, *Biochemistry*, 41, 12043, 2002.
75. Wajant, H., Mundry, K.-W., and Pfizenmaier, K., Molecular cloning of hydroxynitrile lyase from *Sorghum bicolor* (L.). Homologies to serine carboxypeptidases, *Plant Mol. Biol.*, 26, 735, 1994.
76. Trummler, K. and Wajant, H., Molecular cloning of acetone cyanohydrin lyase from flax (*Linum usitatissimum*). Definition of a novel class of hydroxynitrile lyases, *J. Biol. Chem.*, 272, 4770, 1997.
77. Ollis, D.L., et al., The alpha/beta hydrolase fold, *Protein Eng.*, 5, 197, 1992.
78. Cygler, M., et al., Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases and related proteins, *Protein Sci.*, 2, 366, 1993.
79. Wagner, U.G., et al., Mechanism of cyanogenesis: the crystal structure of hydroxynitrile lyase from *Hevea brasiliensis*, *Structure*, 4, 811, 1996.
80. Gruber, K., et al., Atomic resolution crystal structure of hydroxynitrile lyase from *Hevea brasiliensis*, *Biol. Chem.*, 380, 993, 1999.
81. Lauble, H., et al., Structure of hydroxynitrile lyase from *Manihot esculenta* in complex with acetone and chloroacetone: implications for the mechanism of cyanogenesis, *Acta Crystallogr. D Biol. Crystallogr.*, D57, 194, 2001.
82. Lauble, H., et al., Mechanistic aspects of cyanogenesis from active-site mutant Ser80Ala of hydroxynitrile lyase from *Manihot esculenta*, *Protein Sci.*, 10, 1015, 2001.
83. Dreveny, I., Kratky, C., and Gruber, K., The active site of hydroxynitrile lyase from *Prunus amygdalus*: modelling studies provide insights into the mechanism of cyanogenesis, *Protein Sci.*, 11, 292, 2002.
84. Wajant, H. and Pfizenmaier, K., Identification of potential active-site residues in the hydroxynitrile lyase from *Manihot esculenta* by site-directed mutagenesis, *J. Biol. Chem.*, 271, 25830, 1996.
85. Zuegg, J., et al., Three-dimensional structures of enzyme-substrate complexes of the hydroxynitrile lyase from *Hevea brasiliensis*, *Protein Sci.*, 8, 1990, 1999.
86. Gruber, K., et al., Reaction mechanism of hydroxynitrile lyases of the α/β -hydrolase superfamily: the three-dimensional structure of the transient enzyme-substrate complex certifies the crucial role of LYS236, *J. Biol. Chem.*, 279, 20501, 2004.
87. Buehler, H., et al., Enzyme-catalyzed reactions, Part 47. Substrate specificity of mutants of the hydroxynitrile lyase from *Manihot esculenta*, *Chem. Bio.*, 4, 211, 2003.
88. Buehler, H., Miehlisch, B., and Effenberger, F., Inversion of selectivity by applying mutants of the hydroxynitrile lyase from *Manihot esculenta*, *Chem. Chem.*, 6, 711, 2005.
89. Fessner, W.-D. and Helaine, V., Biocatalytic synthesis of hydroxylated natural products using aldolases and related enzymes, *Curr. Opin. Biotechnol.*, 12, 574, 2001.
90. Gotor, V., Biocatalysis applied to the preparation of pharmaceuticals, *Org. Process Res. Dev.*, 6, 420, 2002.

91. Schoemaker, H.E., Mink, D., and Wubbolts, M.G., Dispelling the myths-biocatalysis in industrial synthesis, *Science*, 299, 1694, 2003.
92. Sukumaran, J. and Hanefeld, U., Enantioselective C–C bond synthesis catalysed by enzymes, *Chem. Soc. Rev.*, 34, 530, 2005.
93. Griengl, H., Schwab, H., and Fechter, M., The synthesis of chiral cyanohydrins by oxynitrilases, *Trends Biotechnol.*, 18, 252, 2000.
94. Poechlauer, P., Skranc, W., and Wubbolts, M., The large scale-biocatalytic synthesis of enantio-pure cyanohydrins, in *Asymmetric Catalysis on Industrial Scale*, Blaser, H.U. and Schmidt, E., Eds., Wiley-VCH Verlag, Weinheim, 2004, p. 151.
95. Rosen, T.C. and Dauamann, T., (*S*)-Oxynitrilase: biocatalyst for asymmetric HCN addition, *Chim. Oggi/Chemistry today, Suppl. Chiral Catalysis*, 22(11–12), 21, 2004.
96. Peterson, C.J., et al., Insecticidal activity of cyanohydrin and monoterpenoid compounds, *Molecules*, 5, 648, 2000.
97. Coppola, G.M. and Schuster, H.F., *Chiral α -Hydroxy Acids in Enantioselective Synthesis*, Wiley-VCH Verlag, Weinheim, 1997.
98. Rochowski, A., Untersuchungen zur Säurekatalysierten Hydrolyse von Nitrilen zu Amidinen, Dissertation, Universität Stuttgart, Stuttgart, Germany, 1997.
99. Burkard, U. and Effenberger, F., Enantioselective substitution of 2-(sulfonyloxy)carboxylates with oxygen and sulfur nucleophiles, *Chem. Ber.*, 119, 1594, 1986.
100. Effenberger, F., Burkard, U., and Willfahrt, J., Amino acids. 4. Enantioselective synthesis of N-substituted α -amino carboxylic acids from α -hydroxy carboxylic acids, *Justus Liebigs Ann. Chem.*, 314, 1986.
101. Fleming, P.R. and Sharpless, K.B., Selective transformation of threo-2,3-dihydroxy esters, *J. Org. Chem.*, 56, 2869, 1991.
102. Bousquet, A. and Musolino, A., Hydroxyacetic ester derivatives, namely (*R*)-methyl-2-(sulfonyloxy)-2-(chlorophenyl)acetates, preparation method, and use as synthesis intermediates for clopidogrel, PCT Int. Appl. 1999, WO 9918110 A1, CAN 130:296510, 1999.
103. Sheldon, R.A., et al., The synthesis of angiotensin-converting enzyme (ACE) inhibitors, *Chim. Oggi*, 9, 35, 1991.
104. Weis, R., et al., Erhöhung der Enantioselektivität von Mandel-*R*-HNL durch rationales design des aktiven Zentrums, *Angew. Chem. Int. Ed. Engl.*, 117, 4778, 2005.
105. Kleemann, A. and Engel, J., *Pharmaceutical Substances*, 3rd ed., Thieme, Stuttgart, Germany, 1999.
106. Corey, E.J. and Link, J.O., The first enantioselective syntheses of pure *R*- and *S*-isopreterenol, *Tetrahedron Lett.*, 31, 601, 1990.
107. Enders, D. and Reinhold, U., Diastereo- and enantioselective synthesis of 1,2-amino alcohols and protected α -hydroxy aldehydes from glycol aldehyde hydrazones, *Justus Liebigs Ann. Chem.*, 11, 1996.
108. Li, G., Angert, H.H., and Sharpless, K.B., N-Halocarbamate salts lead to more efficient catalytic asymmetric aminohydroxylation, *Angew. Chem. Int. Ed. Engl.*, 35, 2813, 1996.
109. Bakale, R.P., The development of routes to (*R*)-albuterol hydrochloride, *Spec. Chem.*, 15, 249, 1995.
110. Zandbergen, P., et al., A one-pot reduction–transimination–reduction synthesis of substituted β -ethanolamines from cyanohydrins, *Tetrahedron*, 48, 3977, 1992.
111. Effenberger, F. and Jäger, J., Synthesis of the adrenergic bronchodilators (*R*)-terbutalin and (*S*)-salbutamol from (*R*)-cyanohydrins, *J. Org. Chem.*, 62, 3867, 1997.
112. Brussee, J., et al., Synthesis of optically active ethanolamines, *Tetrahedron*, 46, 1653, 1990.
113. Effenberger, F., Gutterer, B., and Jäger, J., Stereoselective synthesis of (1*R*)- and (1*R*,2*S*)-1-aryl-2-alkylamino alcohols from (*R*)-cyanohydrins, *Tetrahedron Asymmetry*, 8, 459, 1997.
114. Effenberger, F. and Eichhorn, J., Stereoselective synthesis of thienyl and furyl analogues of ephedrine, *Tetrahedron Asymmetry*, 8, 469, 1997.
115. Tromp, R.A., et al., Synthesis of Fmoc-protected (2*S*,3*S*)-2-hydroxy-3-amino acids from furyl substituted chiral cyanohydrin, *Tetrahedron Asymmetry*, 14, 1645, 2003.

116. Skinner, W.A., et al., Topical mosquito repellents. XIII: cyclic analogs of lactic acid, *J. Pharm. Sci.*, 69, 196, 1980.
117. Shapiro, S.L., et al., Pyridylethylated oxazolidinediones. II, *J. Am. Chem. Soc.*, 81, 386, 1959.
118. Fischer, R., et al., Preparation of 3-aryl-4-hydroxy- Δ^3 -dihydrofuranones as pesticides, Ger. Offen. 1995, DE 4337853 A1(1995), CAN, 123, 32947, 1995.
119. Ishikawa, T., Kitajima, J., and Tanaka, Y., Water-soluble constituents of fennel. IV. Menthane-type monoterpenoids and their glycosides, *Chem. Pharm. Bull.*, 46, 1603, 1998.
120. Kobler, C. and Effenberger, F., Enzyme catalyzed reactions. 51. Stereoselective synthesis of *cis-p*-menth-8-ene-1,7-diol, *cis-p*-menthane-1,7-diol and *cis-p*-menthane-1,7,8-triol, *Chem. Eur. J.*, 11, 2783, 2005.
121. Endo, K. and Hikino, H., Structures of regyol, rengyoxide, and rengyolone, new cyclohexylethane derivatives from Forsythia suspensa fruits, *Can. J. Chem.*, 62, 2011, 1984.
122. Ozaki, Y., Rui, J., and Tang, Y.T., Antiinflammatory effect of Forsythia suspensa V(AHL) and its active principle, *Biol. Pharm. Bull.*, 23, 365, 2000.
123. Kobler, C., Bohrer, A., and Effenberger, F., Hydroxynitrile lyase-catalyzed addition of HCN to 2-and 3-substituted cyclohexanones, *Tetrahedron*, 60, 10397, 2004.
124. Effenberger, F., Kobler, C., and Roos, J., Enzyme-catalyzed reactions, Part 44. *cis-trans* selectivity of enzyme-catalyzed additions to 4-substituted cyclohexanones-correlation with the Prelog/Ringold model of enzymatic hydrogenation, *Angew. Chem. Int. Ed.*, 41, 1876, 2002.
125. Effenberger, F., et al., Hydroxynitrile lyase-catalyzed addition of HCN to 4-substituted cyclohexanones; stereoselective preparation of tetronic acids, *Can. J. Chem.*, 80, 671, 2002.

29 Chiral Switches: Problems, Strategies, Opportunities, and Experiences

René Csuk

CONTENTS

29.1	Introduction and Historic Developments.....	699
29.2	Chiral Switch Processes Using Enzymes or Microorganisms.....	704
29.2.1	Dexibuprofen	704
29.2.2	Dexketoprofen = (S)-Ketoprofen	708
29.2.3	Esomeprazole and Pantoprazole	709
29.2.4	Methylphenidate	710
29.2.5	Levofloxacin.....	710
29.2.6	Doxyzosin	711
29.2.7	Other Compounds.....	711
29.3	Concluding Remarks.....	712
	References	713

29.1 INTRODUCTION AND HISTORIC DEVELOPMENTS

Chiral molecules are ubiquitous in nature and have ever-increasing importance in the pharmaceutical industry. This process started with Cushny's observation that one enantiomer of hyoscamine possessed greater pharmacological activity than the other. Thus, chirality of drugs and pharmaceuticals has been in the focus of interest for several decades. The discovery or design and the development of new drugs have been influenced to a great extent by a new understanding of molecular recognition in many pharmacologically relevant events [1–5].

There are several reasons why chiral pharmaceuticals are important. Frequently, the biological activity of one enantiomer is much greater than that of the other, or the other stereomer has no activity at all. Although in some instances the activity of the pure isomer is lower than that of the mixture due to synergistic effects of the isomers, in the worst case, the second isomer can have distinct, even undesirable biological activity [6]. Potential advantages of single-enantiomer drugs [7] reside in a less complex pharmacokinetic profile, a less complex but more selective pharmacodynamic profile, an improved therapeutic index, a reduced potential for complex drug interactions, and finally in a less complex relationship between effects and plasma concentration of the drug.

As these scenarios emerged, it became clear that the failure to address issues raised by the stereochemistry of racemic drugs was the cause of some drug–drug interactions, severe adverse reactions, and withdrawals from the market [4]. From another viewpoint, putting at least nonessential material to patients or into the environment is undesirable and

uneconomic. Thus, producing companies have the opportunity to increase production by a factor of 2 by switching from the generation of a racemic mixture to the production of the pure enantiomer.

Thus, single-enantiomer drugs continue to be a significant force in the global pharmaceutical market. Although the difference in pharmacological activity of enantiomers was known in the early 1900s, it was not until the late 1980s that chirality became a critical issue in the manufacture of agrochemicals and drugs. Interestingly and contrary to common thinking, an enantiomerically pure thalidomide would not have prevented the well-known [4] tragedy. Although thalidomide is the best known example of pharmaceuticals for which one stereoisomer has the desired effect and the other isomer displays harmful properties, other examples include tuberculostatic (*S,S*)-ethambutol where the (*R,R*)-enantiomer causes blindness, or antiarthritic (*S*)-penicillamine, where the (*R*)-enantiomer is extremely toxic. The toxicity of the (*R*) = L-enantiomer to animals including weight loss and death has been known since the 1950s. Whereas in the initial clinical evaluation of the drug in the United States the use of the racemate resulted in severe side effects and was withdrawn, in the United Kingdom (*S*) = D-penicillamine was obtained by a hydrolysis of penicillin as a single enantiomer, and thus the adverse side effects were not observed [8,9].

Similarly, the use of racemic DOPA [10] for the treatment of Parkinson's disease resulted in adverse side effects, whereas the use of L-DOPA resulted in halving the required dose, a reduction of adverse effects, a higher compliance and thus an increased number of improved patients. It was also in the 1980s that the racemate of perhexiline, a drug used to treat abnormal heart rhythms, killed a number of people who had accumulated gram quantities [4] of the wrong stereoisomer impurity which was more slowly metabolized.

Thus, "chiral switches" that exploit one single isomer of an approved racemic mixture are an important feature of drug development [11–14].

In addition to pharmacological benefits, chiral switches-based pharmaceuticals have only limited application in the generic marketplace; they are often intended to allow "line extensions" of blockbuster drugs. Thus, economical bridging strategies have been devised to allow speedy regulatory approval of single enantiomers.

In this concept, chiral switches are chiral drugs that have already been claimed, approved, and marketed as mixtures of enantiomers (or as mixtures of diastereomers), but have been redeveloped as single enantiomers.

The basic definition of a chiral switch can be extended to include chiral drugs that have been sold already as a mixture of diastereomers, but have since been developed as single enantiomers or single-enantiomers E, which have been redeveloped and launched as the paired enantiomer E* [4]. A summary of this approach is depicted in Figure 29.1.

Following standard U.S. laws, a single enantiomer of a previously approved racemic mixture is not considered to be a new chemical entity (NCE) [4,15]. As none of the approved chiral switches was classified as a new molecular entity (defined as an active ingredient that has never been manufactured in the United States), chiral switches are eligible [4,16] for only 3 y exclusivity and are barred from the 5 y of exclusivity that is granted to new drugs.

Currently, there is no single global standard for the development of enantiomerically pure, active pharmaceutical compounds. Producers have to develop these compounds according to the regulatory requirements of each individual country.

Although at present there are only a few agrochemicals sold as single enantiomers, environmental considerations will most likely bring changes. As agrochemicals are usually applied in relatively large amounts compared to drugs for human use, the economic and environmental impacts of a reduced application rate can be significant [4]. Frost and Sullivan [4] forecast that global revenues from chiral technology would grow between 2000 and 2007 at an annual rate of 13% during that period. Agricultural chemicals comprised 14% of the

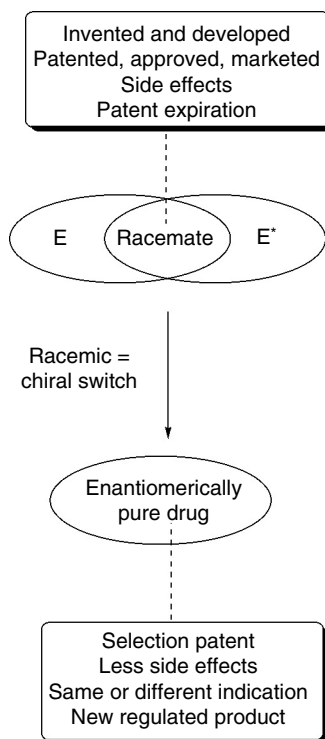


FIGURE 29.1 The chiral switch concept. (Adapted from Agranat, I., Caner, H., and Caldwell, J., *Nat. Rev. Drug Discovery*, 1, 735–768, 2002.)

revenues, while flavors and fragrances accounted for slightly less than 5%. Thus, the most important market for chiral molecules is pharmaceutical drugs.

Single-enantiomer drugs are commanding an ever-increasing presence in the global pharmaceutical market. The worldwide sales of single-enantiomer pharmaceutical products in 2001 was US\$139 billion; the top ten single-enantiomer products resulted in sales of US\$34.2 billion in 2002; in 2003, nine of the top ten drugs contained chiral as the active ingredient, and sales increased to a total of US\$48.3 billion. The chiral switch process has resulted in a number of agents being remarketed as single enantiomers. A summary of those drugs is depicted in Figure 29.2.

In addition to the drugs depicted in Figure 29.2, a number of other compounds are in the pipeline of development [11] or introduction to the market. Among these are (*S*)-oxybutinin (for the treatment of urinary incontinence), (*R,R*)-formoterol (for an improved treatment of asthma), (+)-norcisapride (for the treatment of nocturnal heartburn with a reduction of cardiotoxicity), (*S*)-doxazosin (for the treatment of benign prostatic hyperplasia), (*S*)-amlodipine (for anti-hypertension treatment), (*S*)-fluoxetine (for migraine prophylaxis), as well as (*S*)-lansoprazole and (–)-pantoprazole for the treatment of gastroesophageal reflux.

However, there are some financial risks associated with the development of single enantiomers from racemates. Thus, the termination of the licensing agreement between Sepracor and Eli Lilly [17] for the development of (*R*)-fluoxetine resulted in an estimated loss of ~US\$70 million for Sepracor.

Miconazole, an antifungal agent for the topical treatment of skin diseases, is another candidate for a chiral switch. Although a chiral switch is not needed at all for topical applications, for oral treatment of other diseases, e.g., tuberculosis, a chiral switch has to

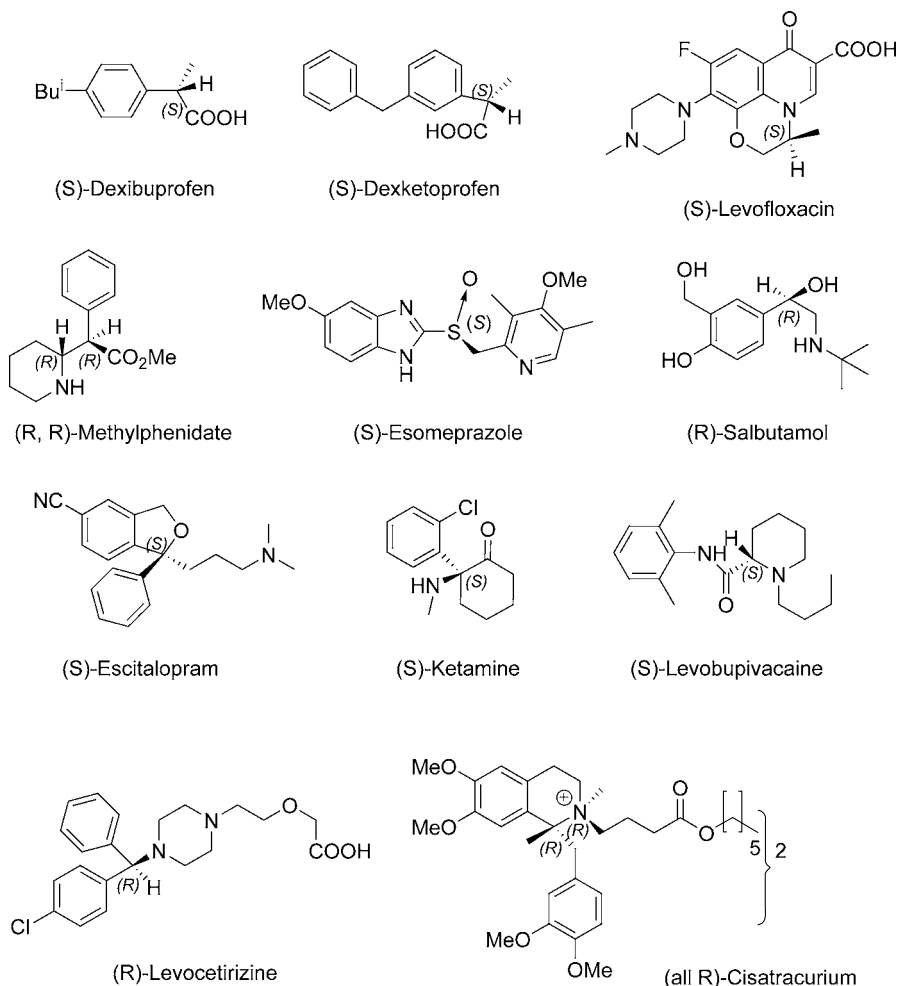


FIGURE 29.2 Marketed single enantiomers of drugs that have undergone the chiral switch. (From Hutt, A.J. and Valentova, J., *Acta Facult. Pharm. Univ. Comeniana*, 50, 7–23, 2003.)

be considered. In addition, several other structurally related antifungal drugs are already taken orally, and some of these are produced in an enantiomerically pure form. Both time and price are determining factors for a successful chiral switch. According to calculations, the additional cost to make one enantiomer of miconazole at 99% enantiomeric excess (ee) would be ~US\$120/kg using advanced simulated-moving-bed (SMB) technology [18]. As the undesired enantiomer cannot be re-racemized, it has to be discarded at the end (thus leading to costs of US\$25/kg).

Several technologies can be used for chiral switches in industrial-scale applications. Among them traditional resolution of racemates still has a leading role, but one has to keep in mind that the cost of resolution rapidly escalates with scale-up. In some cases, SMB technology is cost-effective on a commercial scale. To develop alternative routes to enantiopure materials beginning with racemic raw material will likely be more costly or time-consuming than a chiral-pool approach or the choice of a proper catalytic system. The consensus is that catalytic asymmetric routes are most desirable.

There are only two primary sources for pure enantiomers. The first (and probably oldest) technique involves the isolation of naturally occurring molecules from plants or microorganisms, and, more seldom, from animals. This approach also involves the techniques of *de novo* fermentation of readily available, inexpensive feedstocks. All of these compounds form a “chiral pool.” This chiral pool is therefore a collection of relatively inexpensive, readily available natural products. The main types of chemicals included in the chiral pool are amino and hydroxyl acids, carbohydrates, alkaloids, and terpenes. An “extended chiral pool” also includes newly developed, synthetically derived stereoisomers formed as products, intermediates, or by-products in more or less large-scale commercial processes. The second technique is through synthesis, using optically active, prochiral, or achiral compounds (followed by a suitable process for the separation of the corresponding stereoisomers). These main pathways have been depicted in Figure 29.3.

Many pharmaceuticals have been prepared in an enantiomerically pure form by the crystallization of diastereomeric salts. Among them [19,20] are the antibiotics ampicillin and chloramphenicol (resolved by camphorsulfonic acid), the tuberculostatic ethambutol and the anti-infective thiamphenicol (resolved using tartaric acid), or the antibiotic fosfomycin and the calcium antagonist diltiazem (using *R*-(+)-phenethylamine); the anti-inflammatory agent naproxen, however, has been subject to a classical resolution using the alkaloid cinchonidine.

Crystallization of diastereomeric salts can also involve a process of deracemization. One example [21] includes the conversion of the naturally occurring amino acid L-proline to D-proline using (+)-L-tartaric acid in the presence of a catalytic amount of an aldehyde, finally leading to D-proline (93–95% ee) in up to 95% yield (Figure 29.4). In addition, this methodology can be used for the synthesis of enantiomerically pure compounds starting from the corresponding racemates.

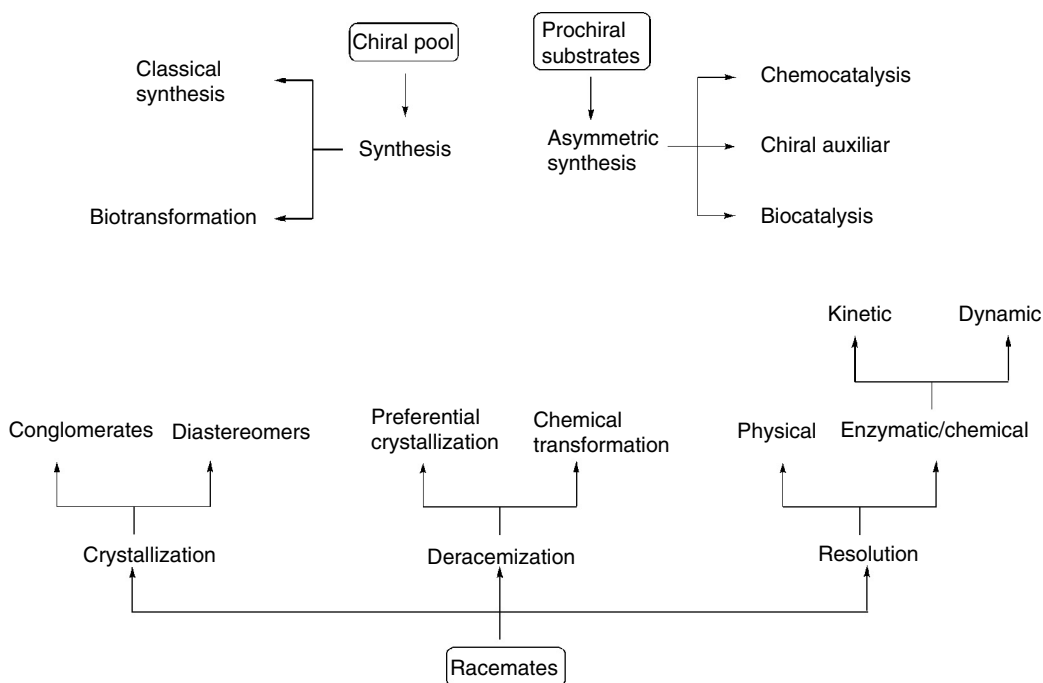


FIGURE 29.3 Main strategies for the synthesis of enantiomerically pure materials.

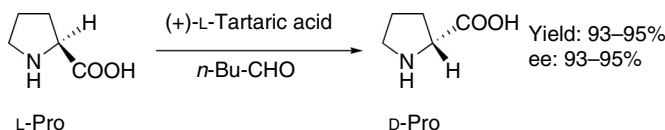


FIGURE 29.4 Deracemization of proline. (From Shiraiwa, T., Shinjo, K., and Kurokawa, H., *Chem. Lett.*, 1413–1414, 1989.)

Contrary to these almost 100% yields resulting from deracemization reactions, kinetic resolutions allow the preparation of enantiomerically pure material, albeit in a maximum yield of 50%. The process of kinetic resolution relies on the differing reactivities of two stereoisomers (preferentially enantiomers in a racemic mixture) with a chiral reagent. One of these stereoisomers reacts more readily than the other and, as a result, two products are obtained: one stereoisomer is converted into a different product and the other remains unchanged. Kinetic resolutions can be achieved with enzymatic, microbial, or chemical methods; an example for the latter is the catalytic Sharpless epoxidation of allylic alcohols [22], as well as several rhodium BINAP [23] catalyzed oxidation processes.

29.2 CHIRAL SWITCH PROCESSES USING ENZYMES OR MICROORGANISMS

Enzymatic kinetic resolutions have been in the focus of interest for the past several years. The use of enzymes (showing some advantages over the use of whole microorganisms) is very attractive because the reactions are often highly selective, can be performed under mild conditions, are catalytic in nature, and, due to the use of water as a solvent, are environmentally benign. No dynamic resolutions have been reported so far for a chiral switch.

29.2.1 DEXIBUPROFEN

α -Arylpropionic acid derivatives form a major class of nonsteroidal anti-inflammatory drugs and household painkillers. Among these, naproxen [2-(4-methoxynaphthyl)-propanoic acid] and ibuprofen [(2-(4-(2-methylpropyl)-phenyl)-propanoic acid] are probably the best known and are sold in large quantities. The sole administration of the (*S*)-enantiomer of ibuprofen is indicated as it has been demonstrated [24] that the (*R*)-enantiomer accumulates in fatty tissue as a glycerol ester whose long-term effects may be critical.

For the synthesis of enantiomerically pure dexibuprofen = (*S*)-ibuprofen, several approaches have been devised using either microorganisms or isolated enzymes. Several key steps have been identified for a straightforward synthesis of this anti-inflammatory drug. Among them, the kinetic resolution of racemic 4-(isobutylphenyl)-propanoic acid in an enzyme membrane reactor led to enantiomerically pure products with an ee >99%. This has been accomplished [25] either by enzymic esterification or enzymic transesterification of the acid with vinyl acetate. The enzyme membrane consisted of a dense surface layer of an asymmetric polyamide capillary membrane combined with an immobilized lipase from *Pseudomonas* sp. It is interesting to note that the immobilized enzyme was significantly more stable than the native; however, its catalytic activity reached only 27% of that of the native lipase. The best resolution of the acid racemate was achieved in the sequential process, starting with an esterification followed by a hydrolysis of the formed ester (Figure 29.5).

An enzyme-facilitated enantioselective transport of (*S*)-ibuprofen through a supported liquid membrane based on ionic liquids [26] has been investigated and ees up to 75% have been obtained. Thus, a hydrophobic isotactic 1-propene homopolymer membrane was used to create a divided cell. One compartment is a feed phase containing racemic ibuprofen and the

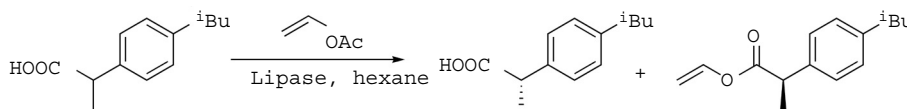


FIGURE 29.5 Dexibuprofen by transesterification. (From Ceynowa, J. and Rauchfleisz, H., *J. Mol. Catal. B Enzymatic*, 23(1), 43–51, 2003.)

lipase from *Candida rugosa* in a buffer–ionic liquid mixture. The receiving phase contained porcine pancreatic lipase (PPL) in a buffer. (*S*)-Ibuprofen is selectively esterified by the *C. rugosa* lipase in the feed phase, the ester dissolves in the ionic liquid phase and permeates across the membrane, and finally (*S*)-ibuprofen ethyl ester is hydrolyzed by the PPL in the receiving phase (Figure 29.6) [26].

The lipase from *C. rugosa* [27] has also been used for the resolution of the corresponding ibuprofen methyl esters (Figure 29.7).

It could be shown that higher conversions were obtained in a fed-batch reactor (compared to a batch reactor), using lower feed rates and high aqueous-phase hold-up. Effects of temperature and the pH value were established, and the reaction also showed some sensitivity to the shaking speed.

As an alternative to *C. rugosa* the use of the carboxypeptidase NP from *Bacillus subtilis* I–85 clones in sorbitan monoleate [28] has been suggested. The stability of the esterase under application conditions was improved by modification with glutaraldehyde, formaldehyde, or glyoxal. The methyl and ethyl esters of ibuprofen have been hydrolyzed by quite a number of different microorganisms [29] such as *Arthrobacter citeus thai*, *Pseudomonas mendocina thai*, *Streptomyces flavovirens*, as well as several strains of *Pseudomonas* and *B. subtilis*. The methyl ester, in addition, has been used [30] to access labeled ibuprofen.

Racemic ibuprofen has been used in another approach [31], but the resolution using PPL has been performed after reduction and acetylation, followed by a reoxidation, thus making this sequence rather lengthy (Figure 29.8).

The ee was high (94%) but both conversion rate (35%) and overall yield low. The same intermediates [32] could be obtained with a different approach using α -methylstyrene oxides (Figure 29.9).

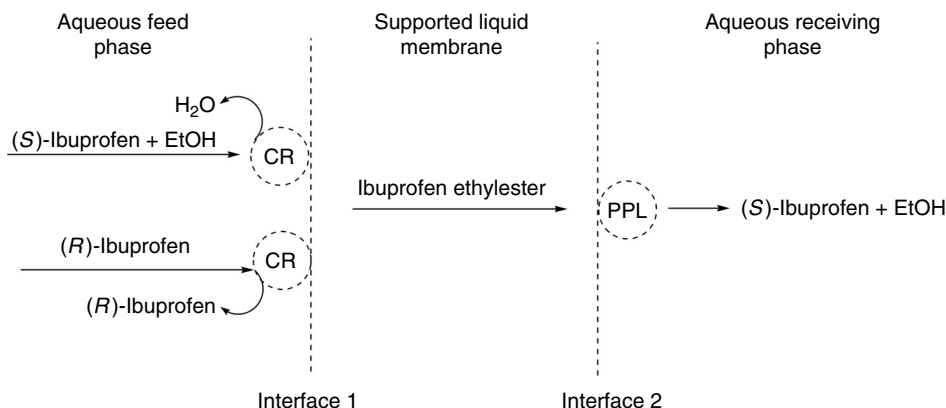


FIGURE 29.6 Dexibuprofen by enantioselective transport. (Adapted from Miyako, E., Maruyama, T., Kamiya, N., and Goto, M., *Chem. Comm.*, 2926–2927, 2003.)

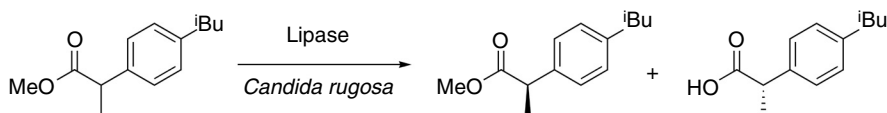


FIGURE 29.7 Dexibuprofen by hydrolysis with the lipase from *Candida rugosa*. (From Madhar, M.V. and Ching, C.B., *J. Chem. Technol. Biotechnol.*, 76, 941–948, 2001.)

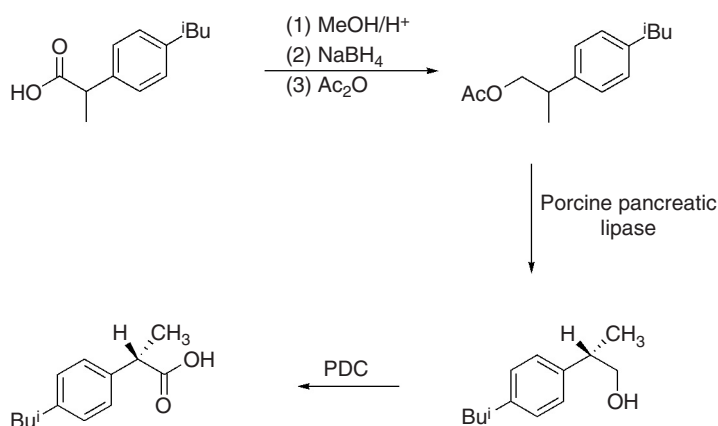


FIGURE 29.8 Dexibuprofen by hydrolysis with the lipase from porcine pancreas. (From Basak, A., Nag, A., Bhattacharya, G., Mandal, S., and Nag, S., *Tetrahedron Asymmetry*, 11, 2403–2407, 2000.)

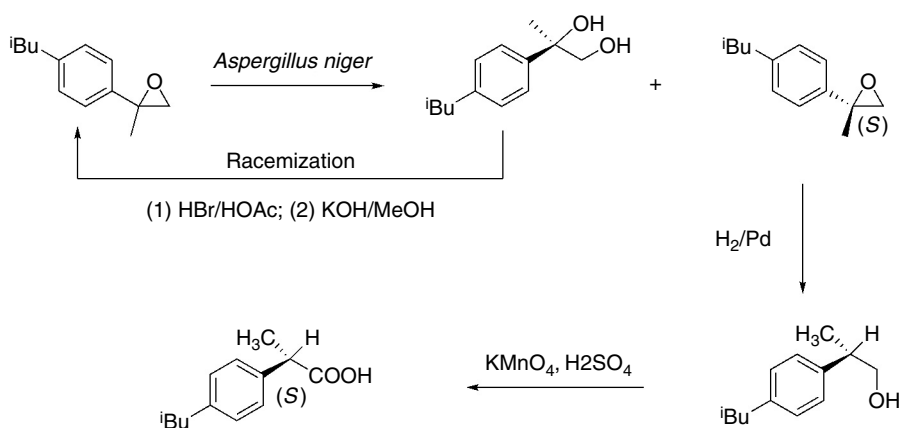


FIGURE 29.9 Dexibuprofen by hydrolysis with *Aspergillus niger*. (From Cleij, M., Archelan, A., and Furstoss, R., *J. Org. Chem.*, 64, 5029–5035, 1999.)

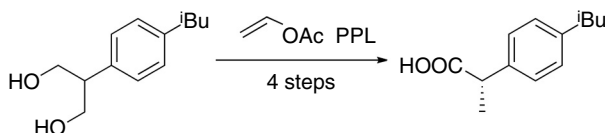


FIGURE 29.10 Dexibuprofen by transesterification. (From Bander, T., Namba, Y., and Shishido, K., *Tetrahedron Asymmetry*, 8, 2159–2165, 1997.)

Using this combined chemoenzymatic strategy (*S*)-ibuprofen was obtained in an optically pure form with a 47% overall yield. The enantioselectivity of the hydrolysis strongly depends on the nature of the enzyme. PPL-mediated transesterification of prochiral 2-aryl-1,3-propanediols seems a rather lengthy [33] approach (Figure 29.10).

In search for higher enantioselectivity in the synthesis of (*S*)-ibuprofen several strategies were followed. New lipases from *Actinomycetes* have been isolated [34] and screened. Several of them displayed higher activity than commercial lipases from *C. rugosa* in the resolution of chiral secondary alcohols. Values of ee that were >99% were obtained using a biocatalyst from *Pseudomonas fluorescens* MTCCB0015, especially when hydrolyzing the 2,2,2-trifluoroethyl ester [35] of ibuprofen. Thus, the (*S*)-acid could be obtained from the corresponding racemic ester after 20 h in 40% isolated yield at 47% conversion. The enzyme lost all of its enantioselectivity using substrates possessing bulky *ortho*-substituents. As far as the lipase from *C. rugosa* is concerned [36], the vinyl ester of racemic ibuprofen is more quickly hydrolyzed [37] than the methyl or chloroethyl ester.

Selectivity of the lipase from *C. rugosa* could be increased by using its cross-linked [38] enzyme crystal (CLEC). CRL-CLEC has been shown to be an attractive replacement for the crude enzyme due to its insolubility (and hence it is easy to recover from the reaction mixture), and these glutaraldehyde cross-linked enzymes are usually 2–3 orders of magnitude more stable than the soluble proteins. Thus, CRL-CLEC has been used for the enantioselective hydrolysis of racemic ibuprofen methyl ester to afford (*S*)-ibuprofen in 87% yield with an ee of 93%.

The influence of temperature [39] and of organic additives, e.g., polar solvents such as *N,N*-dimethylformamide, onto the selectivity of crude lipase from *C. rugosa* for the cleavage of racemic ibuprofen methyl ester has been investigated. As an alternative to the use of soluble enzyme or CLRCs the use of physically adsorbed lipase B from *C. antarctica* was investigated.

It was shown [40] that the reaction yield of the esterification of racemic ibuprofen using adsorbed *C. antarctica* lipase B on an anionic resin could be improved by the addition of benzo[18]crown-6 or metal-tetraphenylporphyrins. While the reaction rate of these reactions was increased, no increase in the enantioselectivity of the enzyme could be observed.

The corresponding nitrile [41] has also been transformed into ibuprofen using immobilized cells. Cells immobilized with both calcium alginate and κ -carrageenan gave a product with lower ee values than for free, intact cells; in addition, the reaction rate was decreased. However, the reaction of cells immobilized in cellulose porous beads with an average diameter of 200 μm containing pores of 10 to 30 μm diameter resulted in improved yields and higher optical purity than those from intact cells. The beads could be used repeatedly without decreasing the optical purity, but the conversion dropped to 32% (Figure 29.11).

Cells from *Rhodococcus butanica* have also been used for the hydrolysis of racemic nitriles, albeit in only 13% yield. The hydrolyses of these α -arylpropionitriles [42] afforded the (*R*)-amides and the corresponding (*S*)-carboxylic acids. A process using an ibuprofen-derived racemic aldehyde or a bisulfite adduct [43] has been described. These procedures utilize

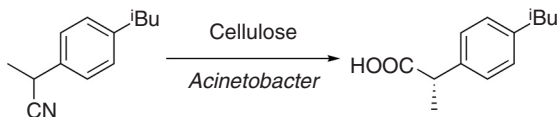


FIGURE 29.11 Dexibuprofen from nitriles. (From Takagi, M., Shirokaze, J.-I., Oishi, K., Otsubo, K., Yamamoto, K., Yoshida, N., and Fujimatsu, I., *J. Ferm. Bioeng.*, 78, 191–193, 1994.)

microorganisms, e.g., *Pseudomonas* sp., *Aeromonas* sp., *Escherichia* sp., *Alcaligenes* sp., or enzyme preparations from these microorganisms containing oxidoreducases (Figure 29.12).

29.2.2 DEXKETOPROFEN = (S)-KETOPROFEN

Lipase catalysis is a well-established method to obtain enantiomerically pure compounds. However, as most of the commercial lipases contain a bunch of competing (iso)enzymes (and also some additives), the enantioselectivity [44] of a lipase is lowered. The enantioselectivity, however, can be increased either by using CLRCs or by increasing the purity of the enzyme [45,46].

The use of crude lipase from *C. rugosa* for the hydrolysis of racemic ketoprofen 2-chloroethyl ester [47] under extremely acidic conditions (pH 2.5) and in the presence of Tween-80 as an emulsifier gave (*S*)-ketoprofen, possessing (after purification) an ee of 95.7% at 42.2% conversion (Figure 29.13).

A purification process has been used for improvement of this approach. Two processes for the partial purification and for the immobilization of the crude lipase preparation (*C. rugosa* lipase OF) have been successfully integrated into one by simple adsorption of the enzyme onto a cation exchange resin (Sephadex) at low pH.

The enantioselectivity was also improved [48] by a partial removal of isoenzymes and by the addition of an emulsifier. Except for Tween-60, Tween-80, and nonylphenol polyethyleneoxy ether, most of the surfactants tested had inhibitory influence on the lipase. Only the addition of Tween-80 [49] resulted in an increase of enantioselectivity. Interestingly, the addition of an enzyme inhibitor [50], e.g., dextromethorphan, enhanced the selectivity of this transformation by a factor of 10. As an alternative, a purification of lipase OF on a mercurial affinity column can be performed, leading to three portions of these enzymes that have remarkably different abilities to differentiate between the enantiomers of α -arylpropionic acids in the lipase-catalyzed hydrolysis of the corresponding esters (Figure 29.14) [48].

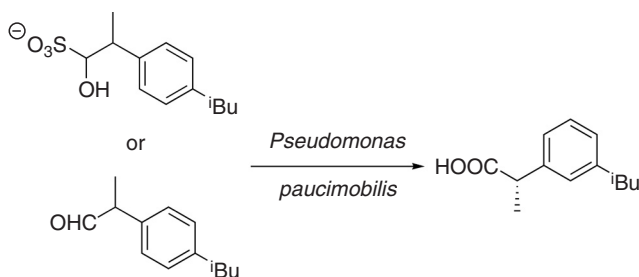


FIGURE 29.12 Dexibuprofen from an aldehyde or its bisulfite adduct. (From Rossi, R.F., Heefner, D.L., and Zepp, C.M., U.S. 5273895 (28.12.1993), *Chem. Abs.*, 120, 215480, 1994.)

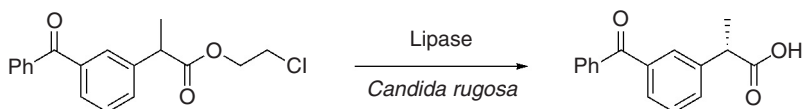


FIGURE 29.13 Dexketoprofen by hydrolysis of its 2-chloroethyl ester. (From Wu, H.-Y., Xu, J.-H., and Liu, Y.-Y., *Synth. Commun.*, 31, 3491–3496, 2001.)

Improved production of (*S*)-ketoprofen has been reported using a mutant of *Trichosporon brassicae* CGMCC0574 (Figure 29.15) [51–54].

The reverse process [55], i.e., the stereoselective esterification, has been accomplished using immobilized lipase from *C. antarctica* (Novozym 435). This process allows the large-scale preparation of (*S*)-ketoprofen with 96% ee as unreacted enantiomer, whereas the (*R*)-enantiomer is recovered as the ester that can easily be separated, racemized by hydrolysis, and be reused in the process.

Finally, some unusual microorganisms [56], e.g., *Aspergillus soje*, *Mycobacterium smegmatis*, *Mucor mihei* [57], *P. fluorescens* MTCCB0015 [58], and acetone powders from liver [59] of rabbit, horse, sheep, dog, etc., have been used.

29.2.3 ESOMEPRAZOLE AND PANTOPRAZOLE

Esomeprazole and pantoprazole are proton pump inhibitors that are indicated for patients with gastroesophageal reflux disease.

Characteristic for this class of compounds are syntheses by microbial redox reactions. Thus, enantiomerically pure or enriched sulfoxides were prepared by the stereoselective biological reduction of racemic sulfoxides. Racemic omeprazole was reacted with *Proteus vulgaris*, reducing (–)-omeprazole [60] to leave (+)-omeprazole in >99% ee.

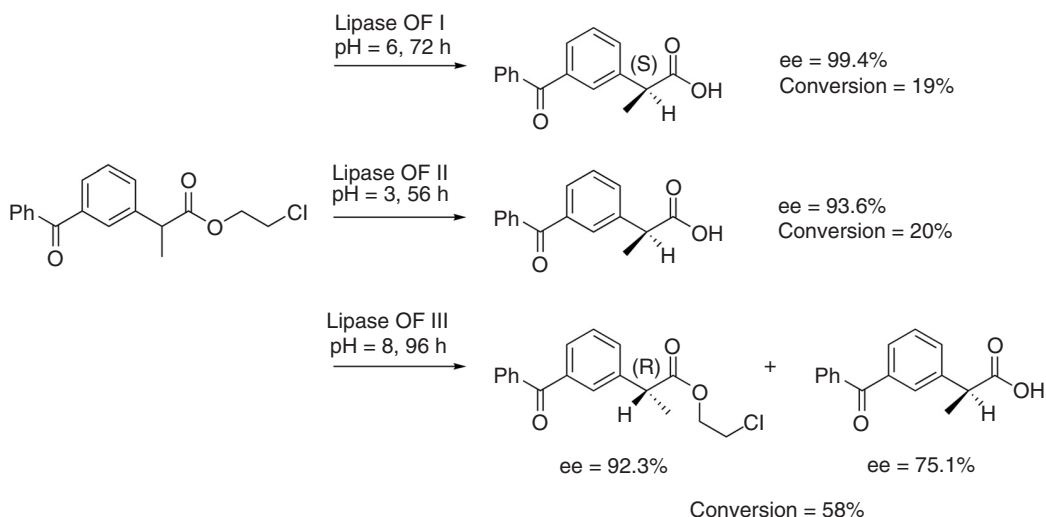


FIGURE 29.14 Dexketoprofen by hydrolysis using purified lipase OF from *Candida rugosa*. (From Liu, Y.-Y., Xu, J.-H., Wu, H.-Y., and Shen, D., *J. Biotechnol.*, 110, 209–217, 2004.)

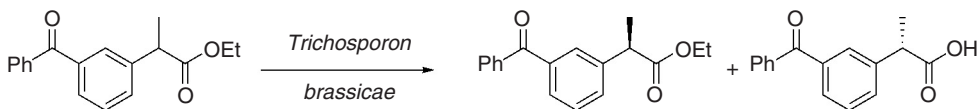


FIGURE 29.15 Dexketoprofen by hydrolysis with *Trichosporon brassicae*. (From Wu, H.-Y., Xu, J.-H., Shen, D., and Xin, Q., *J. Industrial Microbiol. Biotechnol.*, 30, 357–361, 2003; Shen, D., Xu, J., Gong, P., Liu, Y., and Wu, H., *Weishengwuxue Tongbao*, 29, 45–49, 2002; Shen, D., Xu, J.-H., Wu, H.-Y., and Liu, Y.-Y., *J. Mol. Catal. B Enzym.*, 18, 219–224, 2002; Shen, D., Xu, J.-H., Gong, P.-F., Wu, H.-Y., and Liu, Y.-Y., *Can. J. Microbiol.*, 47, 1101–1106, 2001.)

As an alternative, the sulfoxide can be accessed by the microbial oxidation of the corresponding sulfides. Thus (–)-omeprazole was produced in >99% ee by the oxidation of the sulfide [61] with *Penicillium frequentans* or *Ustilago maydis* (Figure 29.16).

29.2.4 METHYLPHENIDATE

(+/-)-threo-Methylphenidate is a mild nervous system-stimulating agent marketed as Ritalin for the treatment of children with attention deficit hyperactivity disorder. The (2*R*,2'*R*)-(+)-threo-derivative has been shown to be several times more active than the corresponding (2*S*,2'*S*)-(–)-threo analog. The enantioselective hydrolysis [62] of the racemate has successfully been performed using α -chymotrypsin in phosphate buffer to yield enantiomerically pure material in ~16% yield. Subtilisin from Carlsberg gave 98% ee (14.5% yield) (Figure 29.17).

29.2.5 LEVOFLOXACIN

Levofloxacin is an antimicrobial. Two strategies have been developed for the synthesis of enantiomerically pure levofloxacin: a resolution at a very early step of the synthetic sequence, and, as an alternative, a resolution at a very late step.

A representative for the first approach was the treatment of racemic lactate derivatives [63] with an esterase to achieve a resolution. In a series of steps the lactate is transformed into the product (Figure 29.18).

A late resolution utilizes immobilized porcine liver esterase (PLE) [64] to cleave ofloxazin butyl ester enantioselectively to levofloxacin (Figure 29.19).

The immobilized esterase (calcium alginate/polyacrylamide gel) exhibited 58% immobilization efficiency, and could be reused five times without severe loss of activity. Significant

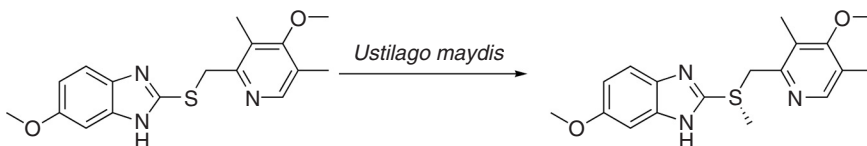


FIGURE 29.16 Esomeprazole by oxidation with *Ustilago maydis*. (From Graham, D., Holt, R., Lindberg, P., and Taylor, S., PCT Int. Appl. 9617077 (28.11.1994), *Chem. Abs.*, 125, 112927, 1996.)

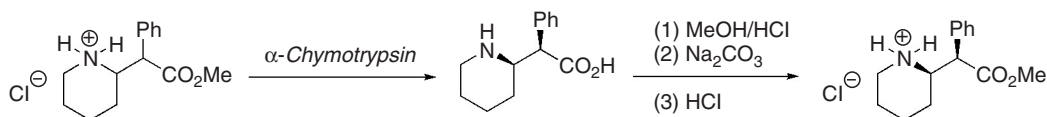


FIGURE 29.17 Methylphenidate by hydrolysis with α -chymotrypsin. (From Prashat, M., Har, D., Repic, O., Blacklock, T.J., and Giannousis, P., *Tetrahedron Asymmetry*, 9, 2133–2136, 1998.)

reduction of the enzyme activity was found, however, when the enzyme was physically adsorbed onto QAE-sephadex.

Similar results were obtained [65] when the enzyme was immobilized in polyacrylamide gel. It was found that the initial activity of the immobilized esterase is significantly affected by the gel composition. The activity of the immobilized enzyme was about 55% compared with that of the free enzyme; the ee was maintained at 60%, which corresponds well to the level obtained with the free enzyme.

In a quite different but rather lengthy approach [66] the use of microorganisms belonging to the genera *Bacillus*, *Micrococcus*, *Actinomycetes*, *Aspergillus*, *Rhizopus*, *Candida*, *Saccharomyces*, and *Zygoascus* has been the subject of a patent.

29.2.6 DOXYZOSIN

The quinazoline-derived compound (+/–)doxyzosin mesylate (Cardura) is indicated for the treatment of hypertension and has been proven effective in the treatment of benign prostatic hyperplasia. Doxazosin seems to be a selective inhibitor of the $\alpha 1$ adrenergic receptor. A very elegant and large-scale approach [67] utilizes a microbial esterase derived from *Serratia marcescens* for the stereoselective hydrolysis of racemic ethyl 1,4-benzodioxan-2-carboxylate to yield ethyl (*S*)-1,4-benzodioxan-2-carboxylate (41 to 43% yield, 95.6 to 98.4% ee) (Figure 29.20).

As an alternative to the use of the esterase from *S. marcescens* the use of the lipase from *C. antarctica* B (Novozyme A/S) has been suggested [68] to obtain the (*S*)-enantiomer with an ee > 95%.

29.2.7 OTHER COMPOUNDS

The development of the antidepressant (*R*)-fluoxetine has been stopped. Nevertheless, a few approaches [69–71] to the target molecule used biotransformations for the synthesis of intermediates. Among them, the stereoselective reduction of ethyl benzoyl acetate with an ee > 99% has been reported using *Geotrichum* sp. or the microorganism genera *Saccharomyces*, *Hansenula*, *Dekkera*, or *Kluyveromyces marxianus*.

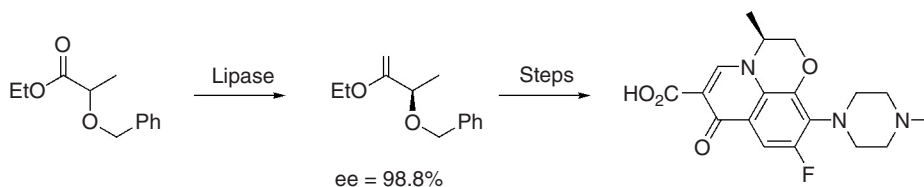


FIGURE 29.18 Levofloxacin from lactate ethers. (From Sato, K., Yagi, T., Kubota, K., and Imura, A., PCT Int. Appl. 2002070726 (12.09.2002), *Chem. Abs.*, 137, 232675, 2002.)



FIGURE 29.19 Levofloxacin by hydrolysis with immobilized porcine liver esterase (PLE). (From Lee, S.-Y., Min, B.-H., Hwang, S.-H., Koo, Y.-M., Lee, C.-K., Song, S.-W., Oh, S.-Y., Lim, S.-M., Kim, S.-L., and Kim, D.-I., *Biotechnol. Lett.*, 23, 1033–1037, 2001.)

No racemic switches for the potent β_2 agonists salbutamol and formoterol utilizing biocatalysis have been established so far. Both compounds are used as bronchodilators in the therapy of asthma and chronic bronchitis. The enantiomers of the local anesthetic bupivacaine exhibit stereoselectivity with respect to blockade of ion channels with the (*R*)-enantiomer being more potent. For this compound, for the selective serotonin re-uptake inhibitor escitalopram, for the anesthetic (*S*)-ketamine, for the H_1 -antihistamine levocetirizine, and for the neuromuscular blocker cisatracurium no enzyme- or microorganism-based chiral switches have been reported so far.

29.3 CONCLUDING REMARKS

Reevaluation of the enantiomers of racemic drugs will continue and result in the introduction of single enantiomers of established drugs into the market. Although this process is quite costly, these costs can be regarded as modest compared to the costs associated with taking a new therapeutic agent to market. In addition, chiral switch approaches provide, at lower costs, drugs of improved efficacy, higher safety, and lower risks due to cleaner pharmacological profiles.

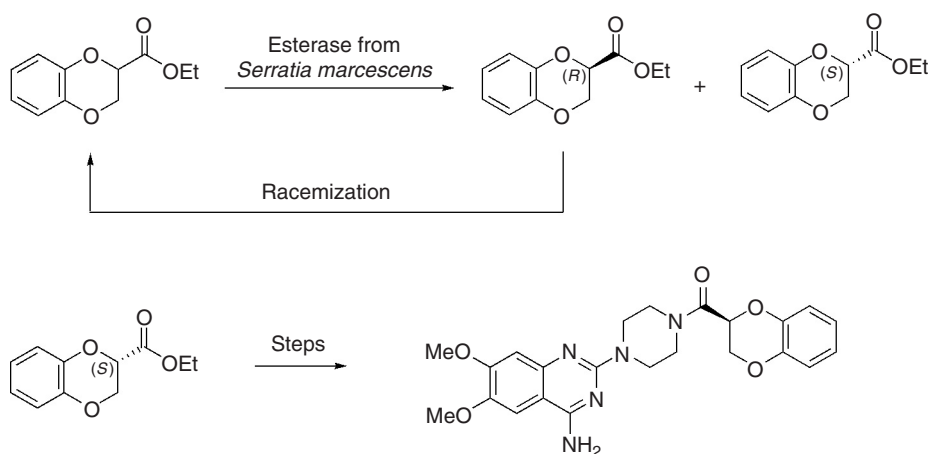


FIGURE 29.20 Doxazosin by hydrolysis using *Serratia marcescens*. (From Fang, Q.K., Grover, P., Han, Z., McConville, F.X., Rossi, R.F., Olsson, D.J., Kessler, D., Wald, S.A., and Senanayake, C.H., *Tetrahedron Asymmetry*, 12, 2169–2174, 2001.)

REFERENCES

1. Eichelbaum, M. and Gross, A., Stereochemical aspects of drug action and disposition, *Adv. Drug Res.*, 28, 1–64, 1996.
2. Triggie, D.J., Stereoselectivity of drug action, *Drug Dis. Today*, 2, 138–147, 1997.
3. Challener, C.A., Ed., *Chiral Drugs*, Ashgate, Burlington, Vermont, 2001.
4. Agranat, I., Caner, H., and Caldwell, J., Putting chirality to work: the strategy of chiral switches, *Nat. Rev. Drug Dis.*, 1, 735–768, 2002.
5. Eichelbaum, M., Testa, B., and Somogyi, A., Eds., *Stereochemical Aspects of Drug Action*, Springer, Heidelberg, 2002.
6. Szelenyi, I., Geisslinger, G., Polymeropoulos, E., Paul, W., Herbst, M., and Brune, K., The real gordian knot: racemic mixture versus pure enantiomers, *Drug News Perspect.*, 11, 139–160, 1998.
7. Hutt, A.J. and Valentova, J., The chiral switch: the development of single enantiomer drugs from racemates, *Acta Facult. Pharm. Univ. Comenianae*, 50, 7–23, 2003.
8. Williams, K.M., Enantiomers in arthritic disorders, *Pharmacol. Ther.*, 46, 273–295, 1990.
9. Walshe, J.M., Chirality of penicillamine, *Lancet*, 339, 254, 2002.
10. Cotzias, G.C., Papavasiliou, P.S., and Gellena, R., Modification of parkinsonism chronic treatment with L-DOPA, *N. Engl. J. Med.*, 280, 337–345, 1969.
11. Agranat, I. and Caner, H., Intellectual property and chirality of drugs, *Drug Dis. Today*, 4, 313–321, 1999.
12. Stinson, S.C., Chiral Switches, *Chem. Eng. News*, 79, 45–56, 2001.
13. Tucker, G.T., Chiral Switches, *Lancet*, 355, 1085–1087, 2000.
14. Rouhi, A.M., Chiral roundup, *Chem. Eng. News*, 80, 43–50, 2002.
15. Strong, M., FDA policy and regulation of stereoisomers: paradigm shift and the future of safer more efficient drugs, *Food Drug Law J.*, 54, 463–487, 1999.
16. Carlson, S.C., The case against market exclusivity for purified enantiomers of approved drugs, *Yale Symp. Law Technol.*, 6, 1999.
17. Thayer, A., Lilly pulls the plug on prozac isomer drug, *Chem. Eng. News*, October 30, p. 8, 2000.
18. Rouhi, A.M., Chiral Chemistry, *Chem. Eng. News*, 82, 47–62, 2004.
19. Bayley, C.R. and Vaidya, N.A., in *Chirality in Industry*, Collins, A.N., Sheldrake, G.N., and Crosby, J., Eds., Wiley, New York, 1992, p. 71.
20. Sheldon, R.A., *Chirotechnology*, Marcel Dekker, New York, 1993, p. 174.
21. Shiraiwa, T., Shinjo, K., and Kurokawa, H., Facile production of (R)-proline by asymmetric transformation of (S)-proline, *Chem. Lett.*, 1413–1414, 1989.
22. Martin, V.S., Woodard, S.S., Katsuki, T., Yamada, Y., Ikeda, M., and Sharpless, K.B., Kinetic resolution of racemic allylic alcohols by enantioselective epoxidation: a route to substances of absolute enantiomeric purity? *J. Am. Chem. Soc.*, 103, 6237–6240, 1981.
23. Kitamura, M., Manabe, K., and Noyori, R., Kinetic resolution of 4-hydroxy-2-cyclopentenone by rhodium-catalyzed asymmetric isomerization, *Tetrahedron Lett.*, 28, 4719–4720, 1987.
24. Williams, K., Day, R., Romualda, K., and Duffield, A., The stereoselective uptake of ibuprofen enantiomers into adipose tissue, *Biochem. Pharmacol.*, 35, 3403–3405, 1986.
25. Ceynowa, J. and Rauchfleisz, H., High enantioselective resolution of racemic 2-arylpropionic acids in an enzyme membrane reactor, *J. Mol. Catal. B Enzym.*, 23(1), 43–51, 2003.
26. Miyako, E., Maruyama, T., Kamiya, N., and Goto, M., Enzyme-facilitated enantioselective transport of (S)-ibuprofen through a supported liquid membrane based on ionic liquids, *Chem. Comm.*, 2926–2927, 2003.
27. Madhar, M.V. and Ching, C.B., Study on the enzymatic hydrolysis of racemic methyl ibuprofen ester, *J. Chem. Technol. Biotechnol.*, 76, 941–948, 2001.
28. Mutsaers, J.H.G.M. and Kooreman, H.J., Preparation of optically pure 2-aryl- and 2-aryloxypropionates by selective enzymatic hydrolysis, *Recl. Trav. Chim. Pays-Bas*, 110, 185–188, 1991.
29. Gist-Brocades, N.V., Manufacture of stereospecific 2-aryl-propionic acids by fermentation, Jpn. Kokai Tokkyo Koho 63045234 (26.02.1988), *Chem. Abs.*, 109, 168–975, 1988.

30. Chen, C.S., Copeland, D., Harriman, S., and Liu, Y.C., Preparation of enantiomerically active deuterium-labeled ibuprofen, *J. Labelled Comp. Radiopharm.*, 28, 1017–1024, 1990.
31. Basak, A., Nag, A., Bhattacharya, G., Mandal, S., and Nag, S., Chemoenzymatic synthesis of antiinflammatory drugs in enantiomerically pure form, *Tetrahedron Asymmetry*, 11, 2403–2407, 2000.
32. Cleij, M., Archelan, A., and Furstoss, R., Microbiological transformations. 43. Epoxide hydrolases as tools for the synthesis of enantiopure α -methylstyrene oxides: a new and efficient synthesis of (S)-ibuprofen, *J. Org. Chem.*, 64, 5029–5035, 1999.
33. Bander, T., Namba, Y., and Shishido, K., Lipase-mediated asymmetric construction of 2-arylpropionic acids: enantiocontrolled synthesis of (S)-naproxen and (S)-ibuprofen, *Tetrahedron Asymmetry*, 8, 2159–2165, 1997.
34. Cardenas, F., Alvarez, E., de Castro-Alvarez, M.S., Sanchez-Montero, J.M., Elson, S., and Sinisterra, J.V., Three new lipases from actinomycetes and their use in organic reactions, *Biocatal. Biotrans.*, 19, 315–329, 2001.
35. Kumar, I., Manju, K., and Jolly, R.S., A new biocatalyst for the preparation of enantiomerically pure 2-arylpropionic acids, *Tetrahedron Asymmetry*, 12, 1431–1434, 2001.
36. Sih, C.J., Gu, Q.M., Fülling, G., Wu, S.H., and Reddy, D.R., The use of microbial enzymes for the synthesis of optically active pharmaceuticals, *Dev. Ind. Microbiol.*, 29, 221–229, 1988.
37. Fülling, G., Schlingmann, M., and Keller, R., Enzymic hydrolysis of 2-arylpropionic vinyl esters for manufacture of the optically active acids, Ger. Offen 3919029 (13.12.1990), *Chem. Abs.*, 114, 245930, 1991.
38. Lalonde, J.J., Govardhan, C., Khalef, N., Martinez, A.G., Visuri, K., and Margolin, A.L., Cross-linked crystals of *Candida rugosa* lipase: highly efficient catalysts for the resolution of chiral esters, *J. Am. Chem. Soc.*, 117, 6845–6852, 1995.
39. Lee, H.W., Kim, K.-J., Kim, M.G., and Lee, S.B., Enzymic resolution of racemic ibuprofen esters: effects of organic cosolvents and temperature, *J. Ferm. Bioeng.*, 80, 613–615, 1995.
40. Gradillas, A., del Campo, C., Sinisterra, J.V., and Llama, E.F., Alteration of the reaction rate in the esterification of (R,S)-ibuprofen by addition of crown ether or porphyrin, *Biotechnol. Lett.*, 18, 85–90, 1996.
41. Takagi, M., Shirokaze, J.-I., Oishi, K., Otsubo, K., Yamamoto, K., Yoshida, N., and Fujimatsu, I., Production of (S)-(+)-ibuprofen with high optical purity from a nitrile compound by cells immobilized on cellulose porous beads, *J. Ferm. Bioeng.*, 78, 191–193, 1994.
42. Kakeya, H., Sakai, N., Sugai, T., and Ohta, H., Microbial hydrolysis as a potent method for the preparation of optically active nitriles, amides and carboxylic acids, *Tetrahedron Lett.*, 32, 1343–1346, 1991.
43. Rossi, R.F., Heefner, D.L., and Zepp, C.M., Enantioselective production of chiral carboxylic acids, U.S. 5273895 (28.12.1993), *Chem. Abs.* 120, 215480, 1994.
44. Chang, R.C., Chou, S.J., and Shaw, J.F., Multiple forms and functions of *Candida rugosa* lipase, *Biotechnol. Appl. Biochem.*, 19, 93–97, 1994.
45. Chang, Y.-F. and Tai, D.-F., Enhancement of the enantioselectivity of lipase OF catalyzed hydrolysis, *Tetrahedron Asymmetry*, 12, 177–179, 2001.
46. Hernaiz, M.J., Sanchez-Montero, J.M., and Sinisterra, J.V., Comparison of the enzymatic activity of commercial and semi-purified lipase from *Candida cylindracea* in the hydrolysis of the esters of (R,S)-2-arylpropionic acids, *Tetrahedron*, 50, 10749–10760, 1994.
47. Wu, H.-Y., Xu, J.-H., and Liu, Y.-Y., A practical enzymic method for preparation of (S)-ketoprofen with a crude *Candida rugosa* lipase, *Synth. Commun.*, 31, 3491–3496, 2001.
48. Liu, Y.-Y., Xu, J.-H., Wu, H.-Y., and Shen, D., Integration of purification with immobilization of *Candida rugosa* lipase for kinetic resolution of racemic ketoprofen, *J. Biotechnol.*, 110, 209–217, 2004.
49. Liu, Y.-Y., Xu, J.-H., and Hu, Y., Enhancing effect of Tween-80 on lipase performance in enantioselective hydrolysis of ketoprofen ester, *J. Mol. Catal. B Enzym.*, 20, 523–529, 2000.

50. Sih, C.J., Improving the enantioselectivity of biocatalytic resolution of racemic compounds, Eur. Pat. Appl. 387068 (12.09.1990), *Chem. Abs.*, 114, 245–954, 1991.
51. Wu, H.-Y., Xu, J.-H., Shen, D., and Xin, Q., Improved production of (*S*)-ketoprofen ester hydrolase by a mutant of *Trichosporon brassicae* CGMCC0574, *J. Ind. Microbiol. Biotechnol.*, 30, 357–361, 2003.
52. Shen, D., Xu, J., Gong, P., Liu, Y., and Wu, H., Isolation of the esterase producer *Trichosporon brassicae* and its catalytic performance in the kinetic resolution of ketoprofen, *Weishengwuxue Tongbao*, 29, 45–49, 2002.
53. Shen, D., Xu, J.-H., Wu, H.-Y., and Liu, Y.-Y., Significantly improved esterase activity of *Trichosporon brassicae* cells for ketoprofen resolution by 2-propanol treatment, *J. Mol. Catal. B Enzym.*, 18, 219–224, 2002.
54. Shen, D., Xu, J.-H., Gong, P.-F., Wu, H.-Y., and Liu, Y.-Y., Isolation of an esterase-producing *Trichosporon brassicae* and its catalytic performance in kinetic resolution of ketoprofen, *Can. J. Microbiol.*, 47, 1101–1106, 2001.
55. D'Antona, N., Lombardi, P., Nicolosi, G., and Salvo, G., Large-scale preparation of enantiopure (*S*)-ketoprofen by biocatalysed kinetic resolution, *Process Biochem.* (Oxford, UK), 38, 373–377, 2002.
56. Iriuchijima, S. and Keiyu, A., Asymmetric hydrolysis of esters by biochemical methods. I. Asymmetric hydrolysis of (+/–)- α -substituted carboxylic acid esters with microorganism, *Agric. Biol. Chem.*, 45, 1389–1392, 1981.
57. Carganico, G., Mauleon Casellas, D., and Palomer Benet, A., A process for the preparation of (*S*)-(+)-2-(3-benzoylphenyl)propionic acid by enzyme-catalyzed enantioselective hydrolysis, PCT Int. Appl. 9325703 (23.12.1993), *Chem. Abs.*, 120, 215–459, 1994.
58. Kumar, I., Manju, K., and Jolly, R.S., A new catalyst for the preparation of enantiomerically pure 2-aryl-propanoic acids. *Tetrahedron Asymmetry*, 12, 1431–1434, 2001.
59. Goswami, A., Stereospecific resolution by hydrolysis of esters of 2-aryl-propionic acids by liver enzymes, PCT Int. Appl. 91131632 (5.9.1991), *Chem. Abs.*, 115, 254–315, 1991.
60. Graham, D., Holt, R., Lindberg, P., and Taylor, S., Enantioselective preparation of pharmaceutically active sulfoxides by bioreduction, PCT Int. Appl. 9617077 (28.11.1994), *Chem. Abs.*, 125, 112927, 1996.
61. Holt, R., Lindberg, P., Reeves, C., and Taylor, S., Enantioselective preparation of pharmaceutically active sulfoxides by biooxidation, PCT Int. Appl. 9617076 (28.11.1994), *Chem. Abs.*, 115, 112–926, 1996.
62. Prashat, M., Har, D., Repic, O., Blacklock, T.J., and Giannousis, P., Enzymatic resolution of (+/–)-threo-methylphenidate, *Tetrahedron Asymmetry*, 9, 2133–2136, 1998.
63. Sato, K., Yagi, T., Kubota, K., and Imura, A., Process for preparation of optically active 2-hydroxypropoxyaniline derivatives as intermediates for levofloxacin via enzymic or microbial stereoselective hydrolysis of racemic lactic acid ester, PCT Int. Appl. 2002070726 (12.09.2002), *Chem. Abs.*, 137, 232675, 2002.
64. Lee, S.-Y., Min, B.-H., Hwang, S.-H., Koo, Y.-M., Lee, C.-K., Song, S.-W., Oh, S.-Y., Lim, S.-M., Kim, S.-L., and Kim, D.-I., Enantioselective production of levofloxacin by immobilized porcine liver esterase, *Biotechnol. Lett.*, 23, 1033–1037, 2001.
65. Lee, S.-Y., Min, B.-H., Song, S.-W., Oh, S.-Y., Lim, S.-M., Kim, S.-L., and Kim, D.-I., Polyacrylamide gel immobilization of porcine liver esterase for the enantioselective production of levofloxacin, *Biotechnol. Bioprocess Eng.*, 6, 179–182, 2001.
66. Sato, K., Takayanagi, Y., Okano, K., Nakayama, K., Imura, A., Itoh, M., Yagi, T., Kobayashi, Y., and Nagai, T., Process for the preparation of benzoxazine derivatives and intermediates therefrom, PCT Int. Appl. 2001018005 (15.03.1991), *Chem. Abs.*, 134, 222719, 2001.
67. Fang, Q.K., Grover, P., Han, Z., McConville, F.X., Rossi, R.F., Olsson, D.J., Kessler, D., Wald, S.A., and Senanayake, C.H., Practical chemical and enzymatic technologies for (*S*)-1,4-benzodioxan-2-carboxypiperazine intermediate in the synthesis of (*S*)-doxazosin mesylate, *Tetrahedron Asymmetry*, 12, 2169–2174, 2001.

68. Kasture, S.M., Varma, R., Kalkote, U.R., Nene, S., and Kulkarni, B.D., Novel enzymatic route for kinetic resolution of (+/–)-1,4-benzodioxan-2-carboxylic acid, *J. Biochem. Eng.*, 27, 66–71, 2005.
69. Schneider, M.P. and Görgens, U., An efficient route to enantiomerically pure antidepressants Tomoxetine, Nisoxetine and Fluoxetine. *Tetrahedron Asymmetry*, 3, 525–528, 1992.
70. Kamal, A., Khanna, G.B., Rao, M.V., and Kondapuram, K.V., Stereoselective preparation of 3-hydroxy-3-phenyl-propionitrile, Int. Pat. Appl. 2002057475 (22.01.2001), *Chem. Abs.*, 137, 108394, 2002.
71. Master, H.E., Newadkar, R.V., Rane, R.A., and Kumar, A., Highly efficient enzymic resolution of homoallyl alcohols leading to a simple synthesis of optically pure fluoxetine and related compounds, *Tetrahedron Lett.*, 37, 9253–9254, 1996.

30 Enzyme Evolution for Chemical Process Applications

Gjalt W. Huisman and James J. Lalonde

CONTENTS

30.1	Introduction	718
30.2	The Biocatalyst Environment.....	718
30.2.1	The Natural Environment.....	718
30.2.1.1	Specificity.....	718
30.2.1.2	Chirality	718
30.2.1.3	Stability.....	719
30.2.1.4	Regulation of Enzyme Activity.....	719
30.2.2	The Industrial Environment.....	719
30.3	Merits and Limitations of Biocatalytic and Whole-Cell Transformations.....	721
30.3.1	Biocatalysts	721
30.3.2	Whole Cells	722
30.4	Methods to Overcome Limitations, Enzyme Improvements.....	722
30.4.1	Enzyme Engineering.....	722
30.4.2	Enzyme Evolution.....	723
30.4.2.1	Enzyme Diversity	724
30.4.2.2	High-Throughput Screening	724
30.4.2.3	DNA Shuffling	724
30.5	Evolving Enzymes for the Process	732
30.6	Applications of Directed Evolution for Biocatalysis.....	734
30.6.1	Organic Solvent Stability	734
30.6.2	Enantioselectivity and Enantiospecificity.....	734
30.6.3	Examples of Evolved Enzymes for Biocatalysis Applications.....	734
30.6.3.1	Amino Acid Oxidase.....	734
30.6.3.2	Hydantoinase.....	735
30.6.3.3	Epoxide Hydrolase.....	735
30.6.3.4	Lipase.....	736
30.6.3.5	Aldolase	737
30.6.3.6	Nitrilase	737
30.6.3.7	Ketoreductase, Glucose Dehydrogenase, and Halohydrin Dehalogenase.....	738
30.7	Conclusion and Prognosis.....	739
	References	739

30.1 INTRODUCTION

The advent of advanced enzyme evolution technologies promises to be the breakthrough technology that will transform biocatalysis into a robust, economically attractive methodology for the manufacture of complex chiral molecules. For many decades, enzymes have been touted as promising catalysts in chemical applications, yet the number of commercial biocatalytic processes has grown only slowly. Recently developed enzyme evolution technologies allow for the rapid development of tailor-made catalysts for commercial processes. This chapter discusses the reasons why such technologies were needed, provides an overview of the different tools that are available and the rationale that guides enzyme evolution, and summarizes recent successes.

30.2 THE BIOCATALYST ENVIRONMENT

30.2.1 THE NATURAL ENVIRONMENT

The “active site” of enzymes consists of amino acid ligands that influence the binding of substrate, product, inhibitors, and activators. In nature, the sequence and type of the amino acids in the polypeptide strands of enzymes have evolved to optimally organize the active site and surrounding ligands such that the function of the enzyme is appropriate for the desired substrates under the right conditions. The environment under which an enzyme functions exerts selective pressure on the structure of the enzyme, specifically the amino acid sequence, providing a competitive advantage to organisms that express an improved enzyme. Within the context of overall fitness of the organism, there are intrinsic limitations on enzymes that are a consequence of the provision of optimal fitness to the host.

30.2.1.1 Specificity

The substrate specificity of an enzyme is a consequence of the role that the enzyme fulfills in cellular metabolism and within the context of alternative substrates that may be prevalent within the cell. For instance, nucleic acid polymerases are either specific for deoxyribonucleotides [DNA polymerase] or ribonucleotides [RNA polymerase]. If this were not the case, DNA could be doped with ribonucleotides and RNA with deoxyribonucleotides with major consequences for the accuracy of DNA replication, transcription, and RNA translation. In nature, DNA and RNA polymerases have ubiquitously evolved to each accept only four nucleotides out of the eight naturally occurring possible substrates as differentiated by the presence of a hydroxyl substituent at the 2' position of the ribose moiety (ribonucleotides) or its absence (deoxyribonucleotides). While the absence of this differentiating moiety is exquisitely well recognized by DNA polymerases, the absence of a hydroxyl group at the 3' position is not and 2',3'-dideoxynucleotides are accepted by DNA polymerase as a substrate. This promiscuity is not a problem for a living cell because dideoxynucleotides are not encountered *in vivo*, but are practically exploited for the development of DNA sequencing *in vitro*.

30.2.1.2 Chirality

Enantioselectivity or enantiospecificity of an enzyme is the key to ensure the reproducibility of cellular events that involve chiral molecules, such as amino acids and proteins. If both D- and L-amino acids were incorporated in a growing polypeptide chain, the population of a given enzyme would be heterogeneous and largely inactive. Naturally, only *S*-amino acids (and glycine) are incorporated into proteins and the whole cellular machinery has adapted to this, so that the undesired amino acid enantiomers are typically not produced, besides a few

(important) exceptions. Thus, natural transaminases, amino acid–ammonia lyases, amino acid synthases, amino acid dehydrogenases, etc. are strictly *S*-specific. Interestingly, the nonnatural *R*-amino acids are frequently encountered in pharmaceutical products, as humans are typically not equipped with enzymes that rapidly clear *R*-amino acid containing molecules. However, for enzymes in catabolic pathways, it may be advantageous not to be enantioselective so that both the enantiomers of an available substrate can be utilized as a source of elements and energy.

30.2.1.3 Stability

Controlled instability of enzymes is extremely important for an organism when it needs to adapt to rapidly changing environmental conditions that require the synthesis of a different set of polypeptides. Protein synthesis is an energy intensive process and synthesis of new enzymes is more efficient—if these building blocks can be salvaged from already produced but superfluous peptides rather than resynthesized from the basic cellular intermediates. Several natural processes have been identified that regulate the stability and hence the half-life of proteins. ppGpp is a global regulator of gene expression in bacteria and is suggested to cause a redirection of transcription so that genes important for starvation survival are favored at the expense of those required for growth and proliferation [1]; protein turnover is catalyzed by a range of different enzymes such as Clp proteases, proteasomes, and aminopeptidases [2–4].

Enzymes are susceptible to environmental conditions, particularly oxidation and certain chemical instabilities, for instance, aspartate isomerization. In nature, such degenerated polypeptides are removed from circulation by proteolytic system [5]. Although some enzymes from nature may be stable for days, the typical half-life of natural biocatalysts is in the range of minutes [6], which is typically insufficient for an industrial chemical process.

30.2.1.4 Regulation of Enzyme Activity

It is crucial for a cell to regulate the relative activity of enzymes, particularly as they operate in pathways or regulons. While randomly scavenging available nutrients may appear as an opportune approach for an organism in dealing with a plethora of substrates, such uncontrolled action would put a burden on the cellular machinery with the need to synthesize a broad range of catabolic enzymes. For efficiency reasons, utilization of preferred substrates over less-desired nutrient sources is facilitated in organisms by mechanisms such as induction and repression at the gene level (e.g., induction of the *lac* operon by lactose and repression by glucose), isozyme availability at the regulon level (e.g., three different isozymes for the common first-step in the synthesis of methionine, threonine, and lysine from aspartate), and substrate/product inhibitions at the enzyme level, (e.g., phosphofructokinase and fructose-1,6-bisphosphatase) [7,8]. Feedback control mechanisms, such as substrate and product inhibitions, are important in biological systems but limit the utility of enzymes in chemical manufacturing processes.

30.2.2 THE INDUSTRIAL ENVIRONMENT

The environment that an enzyme encounters during a chemical process is significantly different from that in a biological environment. Where metabolite concentrations in living cells are typically <10 mM [9] and are subjected to natural homeostasis mechanisms such as product inhibition of enzymes involved in metabolism, concentrations in chemical processes are orders of magnitude higher and as a consequence, the biocatalyst must function over a large concentration range. For a commercial chemical process, economic performance is

key and product concentrations of at least 10 to 15% (w/v) are generally required, which often correspond to concentrations in excess of 500 mM. As a result, substrate conversion to product can lead to significant changes in the environment that the enzyme encounters (Figure 30.1). For instance, at 1 M substrate concentrations, enzyme-catalyzed reactions may produce 1 M of additional ionic strength in the form of an acid salt (in a lipase-catalyzed reaction), chloride (in a dehalogenase-catalyzed reaction), and gluconate (in a ketoreductase/glucose dehydrogenase cofactor regeneration system) with a potential destructive effect on the enzymes. Such changes in osmotic strength of the reaction medium are not encountered in nature and enzymes have not evolved naturally for such fluctuations. The development of glucose dehydrogenase variants, which are less sensitive to high salt concentrations, is an example of how new protein engineering technologies have begun to address such issues [10].

The substrates for biocatalytic transformations are frequently hydrophobic, leading to the presence of a secondary phase in the reaction where the enzyme functions at the organic–aqueous interface. Usually, the substrate of interest is so poorly soluble that organic cosolvents can be required to afford appreciable reaction phase concentrations of the substrate. As enzymes in nature do not typically encounter large amounts of organic substrates or solvents, this adds physical stress to the enzyme. Given that the concentration of organics in water is dependent, among others, on the makeup of the aqueous phase, the changing ionic strength in the medium can greatly alter the organic substrate availability over the course of a reaction.

Natural regulation mechanisms for enzyme activity, such as substrate and product inhibition, are important to protect the cellular environment from toxic levels of metabolites, yet in a chemical process, the volumetric productivity and the ease of process operation depend on fast and complete conversion of all substrate. With the need for high conversions in short periods of time, substrate and product inhibition are a major problem for industrial application of enzymes.

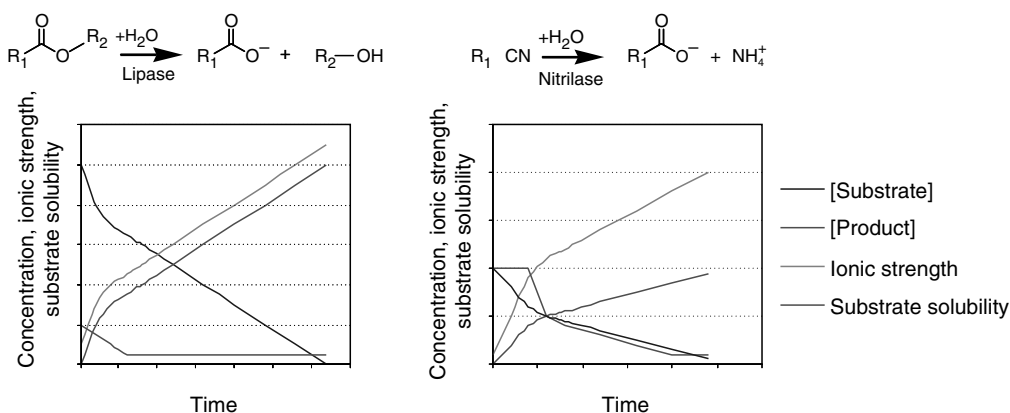


FIGURE 30.1 Environmental changes in a chemical process to which an enzyme may be exposed. (a) Lipase reaction in 100 mM buffer at $t = 0$. With substrate available at 1 M, the ionic strength at the end of the reaction is >1 M. With the increase in osmotic strength, the substrate solubility (accessibility) may diminish resulting in a slower reaction. (b) Nitrilase reaction in 100 mM buffer at $t = 0$. The ionic strength of the reaction medium increases quickly with two ionic products. In addition, the organic substrate is converted to a fatty acid, which at the critical micelle concentration leads to partitioning of substrate and product.

30.3 MERITS AND LIMITATIONS OF BIOCATALYTIC AND WHOLE-CELL TRANSFORMATIONS

Historically, both (semi) purified enzymes and whole cells (microbial/plant/mammalian) have been used in chemical conversions. Either system has advantages and disadvantages and can in principle be selected for process development. Before we examine the shortcomings of these systems, it is important to consider all aspects of a commercial biocatalytic process, including the conditions that the catalyst is subjected to prior and subsequent to chemical conversion.

1. The biocatalytic process starts with catalyst manufacture, generally by fermentation of a natural or recombinant microbial strain.
2. At the end of fermentation, the biomass is used directly or is harvested and formulated as whole-cell catalyst.
3. The enzyme is separated from other cell constituents in a down-stream process and formulated. In considering the different options for formulation, storage and transport are important factors.
 - If the catalyst is used at the same site as where it was produced, a catalyst suspension can directly be loaded into the chemical reactor or the reactants can be added to the fermenter where the catalyst was produced.
 - When catalyst manufacture is tolled to a site different from the chemical plant, the catalyst needs to be transported under controlled conditions and volume minimization (e.g., removal of water by lyophilization) may be required.
 - Upon arrival, the material may need to be stored for variable periods of time and prepared prior to use.
4. At the end of the biocatalytic reaction, the product is isolated and removal of all biologic material is important. Especially in the synthesis of pharmaceutical end products, the absence of proteinaceous material and endotoxins is key as these molecules carry the potential to cause significant side effects (such as allergies and other immunity related side effects).

When large catalyst-to-substrate ratios are needed to accomplish the desired reaction (low substrate-to-catalyst ratio), the separation of product and catalyst becomes increasingly difficult due to emulsion formation and isolated yield of product is compromised. This short overview indicates some general phenomena that are important for biocatalysts, whether the application is as whole cells or formulated enzyme: manufacturability, formulation options, stability during production, long-term storage stability, compatibility with the chemical reaction conditions, and separation from the chemical product.

30.3.1 BIOCATALYSTS

In pure form, enzymes often exhibit longevity and are easy to store and transport. The early purification and removal of biological contaminants facilitate the work-up of the chemical process. Biocatalytic processes are much more adaptable than whole-cell processes, as the function of the catalyst is not deterred by the limitations that other components of a whole cell may exert. Consequently, more degrees of freedom in process design are available, including substrate concentration, pH, temperature, nature of organic solvent, and use of immobilization matrices if desired. However, the use of free enzymes in chemical reactions

is typically limited by the availability of commercial enzymes with the desired activity on the substrate of interest. Many of these enzymes have been developed for use in the food or detergent industry and thus may not be suitable for chemical manufacture. Even if a commercial enzyme is found to have activity on a target substrate, frequently there is insufficient activity to make the process economically viable. In cases where an enzyme is identified that performs the chemistry well, application at industrial scale is frequently hampered by insufficient stability. Frequently applied engineering solutions such as stabilization by cross-linking, immobilization (with or without recycling of the catalyst), substrate engineering, solvent change, and continuous product extraction have sometimes been implemented at scale. In reactions that involve the stoichiometric use of cosubstrates, such as NAD(P)H, the efficient use of these molecules by regeneration is crucial for the economics of the process. This puts additional requirements on the enzymes in terms of affinity for the cosubstrate as well as an activity requirement, because the enzymatic reaction needs to be completed before the intrinsic instability of cosubstrate impedes the reaction.

30.3.2 WHOLE CELLS

For whole-cell reductions, cosubstrate regeneration is not an issue as the cell is naturally equipped for this. Microbes have adapted to the natural environment and produce both simple and complex metabolic products from their nutrient sources through complex, integrated pathways. Whole cells can be used directly in chemical processes thereby greatly minimizing the formulation costs. Whole cells are cheap to produce and no knowledge of genetic details is required.

However, processes that use whole organisms as catalyst are limited due to the intrinsic need to grow biomass, which results in the formation of by-products that need to be removed during the work-up after the chemical step. Both the cells themselves and the wide range of typical metabolites are potential process impurities. Although the use of filtrations and liquid extractions can limit the contamination of the product, these procedures add significant cost. Additionally, if it is required that the catalyst is intact during the process (e.g., during reactions that require cofactor regeneration), the survival boundaries of the cell limit the chemical process conditions. Another limitation is the short lifetime of whole-cell catalysts and the relatively long time required for the biomass production. Typically, biotransformations or fermentative processes are slow and thus require significant production capacity. As in biocatalytic processes, engineering solutions have been sought for some of the problems encountered with whole-cell processes with some success. The manufacture of acrylamide at 1000s mT scale using immobilized whole cells of a *Rhodococcus* strain is a great example [11]. However, when no engineering solutions exist, improving these catalysts is difficult due to the complexity of whole cells and the inherent problems with adapting the physiology of a cell.

30.4 METHODS TO OVERCOME LIMITATIONS, ENZYME IMPROVEMENTS

30.4.1 ENZYME ENGINEERING

With the advent of recombinant DNA technology in the early 1970s, the number of enzymes available for biocatalytic studies and applications increased as enzymes no longer needed to be isolated from their natural source. Genes encoding enzymes of interest were frequently overexpressed in *Escherichia coli*, with mixed success. As these tools became more established, the number of recombinantly produced enzymes continued to climb. In the 1980s, tools had advanced and hence knowledge of the three-dimensional structure of an enzyme could be

used to engineer enzymes with high precision at the molecular level. Although initial technical results were promising, the speculated increase in the potential of enzymatic conversion was not fully realized; primarily due to the expense of protein structure determination and the complexity of enzymes that limits the full understanding of how enzymes function at a molecular level, let alone under different environmental conditions. Recognizing the power of recombination in generating variety among antibodies, Stemmer invented what became the first of many recombination-based *in vitro* laboratory evolution methods, DNA shuffling [12]. His landmark paper initiated an added interest in the field of biocatalysis and led to the development of several *in vitro* and *in vivo* evolution methods.

30.4.2 ENZYME EVOLUTION

Given that the starting, natural enzyme is typically not sufficiently “fit” for a chemical process, several rounds or iterations of library generation and high-throughput (HTP) screening are generally required. “Fitness” is frequently used in the enzyme evolution field as a term that encompasses all the enzyme properties desired for large-scale application. Properties such as specific activity, substrate and/or product inhibition, tolerance to organic solvents, thermostability, oxidative stability, stability to high and low ionic strength, storage stability together are parameters in the fitness function. Accordingly, in enzyme evolution it is desirable to address as many of these parameters during the HTP screens as possible, so that no variants are taken forward that are deteriorated for any of these parameters. The essence of such an approach can be summarized in a relatively simple adage “you get what you screen for.” While this indicates that knowing what the desired enzymatic properties are is key, oversimplification should be cautioned against as often you may “lose what you have, if you do not screen for it” as can be observed in the literature [13].

In vitro laboratory, enzyme evolution is comprised of three key components that form an iterative process until the desired enzyme has been identified (Figure 30.2):

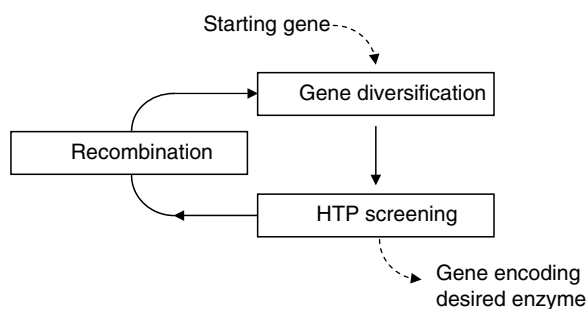


FIGURE 30.2 The evolution cycle. The gene of interest is diversified by procedures as described in the text, resulting in mutations that have been preselected in nature (e.g., homologous enzymes), or mutations that are novel, such as by error-prone PCR. Resulting libraries are screened and enzyme variants with improved properties are identified. If further improvement of the best variants is desired, the corresponding genes are recombined (optionally including additional diversity) and rescreened. It is desirable that the HTP screen resembles the ultimate process conditions as closely as possible so that the most attractive process can be enabled. The most informative screens tend to require larger reaction volumes than less-informative screens, and consequently, the throughput may be compromised. A reduced throughput can be tolerated, however, if the quality of the gene libraries is high, i.e., the chance of finding improved variants is high. The different gene evolution technologies described in this chapter provide gene libraries of different quality, each requiring different levels of throughput.

1. The generation of diverse enzyme libraries
2. The identification of improved variants in such libraries using HTP screening
3. The recombination of beneficial mutations to generate diverse enzyme libraries that are further improved for desired function

All three components are of equal importance. In the absence of “high-quality” diversity, “highly relevant” screens, or “efficient” recombination, the generation and identification of enzyme variants becomes difficult, thereby decreasing the overall pace of the *in vitro* evolution process.

30.4.2.1 Enzyme Diversity

Large-scale genome sequencing efforts have provided a wealth of diverse enzyme sequences. The number of publicly available microbial genomes for which the complete DNA sequence has been determined is approaching 250 [14]. Genes encoding enzymes that have evolved for optimal fitness in the natural environment are thus abundantly available. However, as discussed above, such enzymes are rarely adept at functioning well in a chemical plant. Methods to generate nonnatural variants have been developed and include classical methods such as error-prone polymerase chain reaction (PCR), site directed mutagenesis, cassette mutagenesis, as well as more sophisticated methods that are specific [15] or random, such as “deletion-duplication” [16] and “sequence saturation mutagenesis” [17].

30.4.2.2 High-Throughput Screening

HTP screening equipments for the identification of improved enzymes are widely available as stand-alone units or are combined with other instruments in robotic setups. In some instances, commercially available equipment can be rebuilt for specific applications in HTP screening for improved enzymes.

The generally used approach in HTP screening for improved enzymes is a tiered protocol, where the number of variants under analysis is quickly reduced through successive screens of decreasing throughput, providing information of increasing relevance. For instance, first tier screens can be microbiological selections (TP: $\sim 10^9$), fluorescence-assisted cell sorting (TP: $\sim 10^9$), plate assays on bacterial colonies, e.g., for lipases, proteases, etc. (TP: $\sim 10^4$); second tier screens can be 96-well kinetic assays (TP: $\sim 10^3$), and third tier screens can be chemical reactions at a 25 to 100 ml scale (TP: $\sim 10^0$ /day).

30.4.2.3 DNA Shuffling

The iterative process of gene diversification, library construction, and HTP screening comprise the enzyme evolution cycle depicted in [Figure 30.2](#). In the present traditional approach [12], a number of gene variants that encode enzymes with improved function would enter the next round of evolution to further improve fitness. The understanding of the power of such genetic algorithms was recognized in the engineering disciplines, in the 1950s and was applied almost universally in complicated construction and engineering problems since then, but not in the protein engineering field. Recently, the importance of iterative homologous recombination for sequence evolution was underscored by *in silico* simulations [18]. With the advent of bioinformatics tools [19], this paradigm has shifted in that the single best evolved variant of each round is designated as the “backbone” for further libraries, which are then generated by introducing a set of mutations that were identified in other “hits.” While the traditional approach advances genes and mutants into next rounds of evolution, these newer methods advance mutations into subsequent

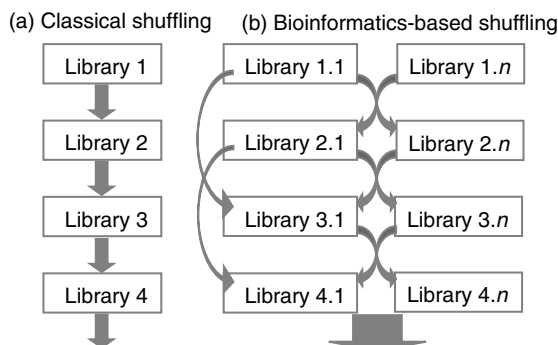


FIGURE 30.3 Evolution of enzyme evolution approaches. (a) Traditional shuffling approach—a few “hits” are taken forward from each library in consecutive, serial rounds. Diversity is incorporated at the beginning and is derived from random mutagenesis, recombination, and homologous genes. (b) Bioinformatics aided shuffling approach—beneficial and neutral diversities are taken forward from parallel libraries ($n = 1\text{--}20$) spanning multiple rounds. Diversity is incorporated throughout and derived from random mutagenesis, saturation mutagenesis, homologous sequences, and structural information.

rounds (Figure 30.3). One of the additional advantages of this method is that mutations can be added more freely into several, parallel library designs and at different times during the evolution program. In fact, individual mutations can be introduced into library generation, even if they were identified much earlier in the program or in different projects that utilized the same genetic information.

The following section provides a synopsis of some evolution methods that involve a recombination step, but is by no means a complete summary. Several recent reviews are also available for the interested reader [20–23].

30.4.2.3.1 Gene Shuffling (Figure 30.4)

Method description: This is a method for *in vitro* homologous recombination of pools of selected mutant genes by random fragmentation and PCR reassembly.

Example: β -Lactamase was evolved in three cycles of mutagenic DNA shuffling and two cycles of backcrossing with wild-type DNA, each round followed by selection on increasing concentrations of the antibiotic cefotaxime. The minimum inhibitory concentration was increased 32,000-fold, while nonrecombinatory procedures such as cassette mutagenesis and error-prone PCR resulted in only a 16-fold increase. [12]

Note: The revolutionary method that changed the field of enzyme engineering.

Semisynthetic Shuffling (Figure 30.5)

Method description: Genes and plasmids were reassembled from DNA fragments 10 to 50 bp in size. Complete recombination was obtained between two markers on two genes separated by 75 bp. Oligonucleotides could be incorporated into the reassembled gene to target specific regions.

Example: A library of chimeras of the human and murine genes for interleukin 1 beta was prepared [24].

Note: The first account of the use of synthetic oligonucleotides in recombination-based enzyme engineering.

Low Mutageneses DNA Shuffling

Method description: An alternative DNA shuffling protocol for random recombination of homologous genes *in vitro* with a low rate of point mutagenesis (0.05%). The mutagenesis rate is controlled over a wide range by the inclusion of Mn^{2+} or Mg^{2+} during DNase I digestion,

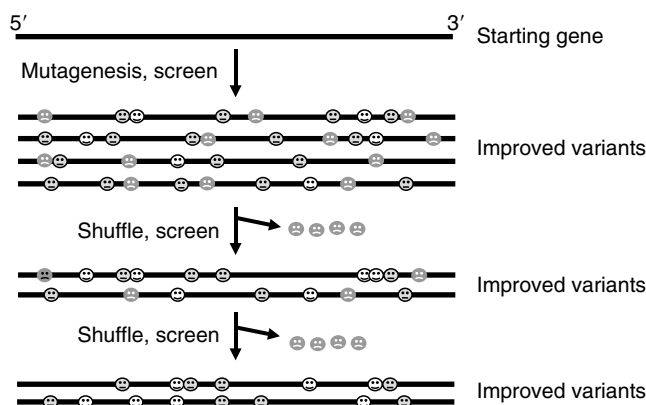


FIGURE 30.4 Single gene shuffling. A single gene is improved in iterative cycles of recombination and screening. Initial diversity is created by mutagenesis, such as error-prone PCR. This random method creates mutations that are desired (good mutations—happy faces), undesired (bad mutations—sad faces) and those that are neutral. Evolution methods that involve iterative mutagenesis of improved variants accumulate all three types of mutations and overall improvement is limited by the extent that the desired mutations can offset the negative effects of the undesired mutations. In recombination-based methods, the undesired mutations are removed so that the extent of possible improvement is not limited by undesired mutations.

by choice of DNA polymerase used during gene reassembly as well as how the genes are prepared for shuffling (PCR amplification vs. restriction enzyme digestion of plasmid DNA).

Example: Subtilisin E and a thermostable mutant were shuffled to demonstrate that DNA shuffling can be achieved with high precision and that closely linked mutations can be separated during the protocol [25].

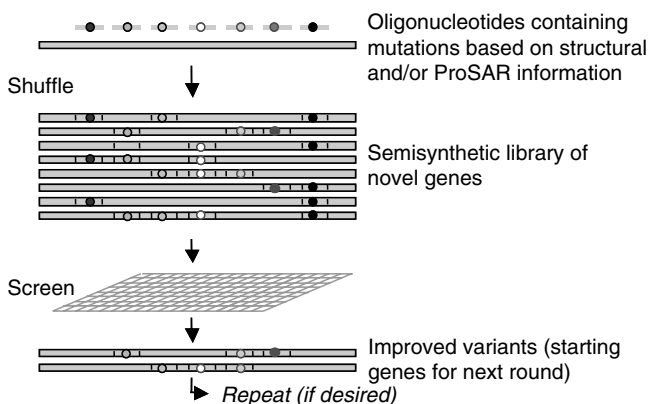


FIGURE 30.5 (See color insert following page 526) Semisynthetic shuffling. The nature of the genetic diversity that is recombined with the gene of interest is biased using oligonucleotides that contain specific mutations that are expected to be beneficial. Such mutations can be based on structural knowledge of the enzyme, hot spots in the gene found to be of interest in earlier rounds of evolution (even from different projects that utilized the same starting gene), or mutations that have been identified by computational methods as desirable. The level of incorporation of the oligonucleotides is controlled experimentally to create semisynthetic gene libraries with a variable number of mutations.

Staggered Extension Process (StEP)

Method description: This method consists of priming the template sequence(s) followed by repeated cycles of denaturation and extremely abbreviated annealing/polymerase-catalyzed extension. In each cycle, the growing fragments anneal to different templates based on sequence complementarity and extend further. This is repeated until full-length sequences have formed. Due to template switching, most of the polynucleotides contain sequence information from different parental sequences.

Example: A set of five thermostable subtilisin E variants were recombined and yielded an enzyme whose half-life at 65°C is 50 times that of wild-type subtilisin E [26].

Note: An alternative to the methods that use enzymatic, chemical or physical DNA fragmentation.

Heteroduplex Formation

Method description: Heteroduplexes prepared *in vitro* can be used to transform bacterial cell where they are repaired to form recombinant genes composed of elements from each parent.

Example: The method is demonstrated using GFP as model system [27].

Note: A laborious procedure that does not seem to be used frequently.

Direct Transformation of PCR Products

Method description: Libraries of shuffled gene PCR products are directly transformed into *Bacillus subtilis* or *Acinetobacter calcoaceticus*. Reconstitution of a selectable marker provides a simple selection for evolved genes [28].

Note: The transformation efficiency is rather low such that the library production may be problematic.

Functional Salvage Screen

Method description: The activity of nonfunctional enzymes is restored by random cloning of genomic DNA fragments in a predetermined region of the gene of interest.

Example: The method is demonstrated using GFP [29].

Note: An interesting approach that may be of value when structural elements need replacement, but due to the disruptive character of the method, ultra HTP screens or selections are needed, limiting the overall applicability of the method.

Synthetic Shuffling

Method description: The variety in functional libraries is increased by using degenerate oligonucleotides; starting genes are no longer necessary and codon usage criteria can be incorporated as well.

Example: Synthetically shuffled libraries of 15 subtilisin genes were generated, yielding active and highly chimeric enzymes with desirable combinations of properties that were not obtained by other directed-evolution methods [30].

Note: This paper shows the versatility of shuffling approaches varying from natural genes as starting materials to completely synthetic genes.

EndoV Fragmentation

Method description: Endonuclease V nicks uracil-containing DNA at the second or third phosphodiester bond 3' to uracil sites and is used to randomly fragment DNA. Cleavage occurs at random sites and the length of the fragments can be adjusted by varying the concentration of dUTP in the PCR. Unlike the DNase I methods, no partial digestion or gel separation of fragments is required.

Example: Two truncated GFP genes were recombined [31].

Note: An alternative method for DNA fragmentation.

Mutagenic and Unidirectional Reassembly (MURA)

Method description: This method generates libraries of DNA-shuffled and randomly truncated proteins. The method involves random fragmentation of the template gene(s) and

PCR reassembly with a unidirectional primer. The MURA products were treated with T4 DNA polymerase and subsequently with a restriction enzyme whose site was located on the region of the MURA primer.

Example: Phospholipase was converted into variants that exhibited absolute lipase activity by truncation at a region beginning with amino acids 61 to 71, together with amino acid substitutions [32].

Note: A variant library manufacturing technique.

Computational Prescreen

Method description: A combined computational and experimental method for the rapid optimization of proteins

Example: The active site of β -lactamase was redesigned using a computational screen. The screen eliminated sequences that were incompatible with the protein fold, thereby reducing the number of sequences that need to be screened experimentally. In a single round, 1280-fold improved variants were obtained from an antibiotic resistance selection. None of the mutations had been observed before in either natural or nonnatural β -lactamases [33].

Note: Even after size reduction, the experimentally screened library is very large and requires ultra HTP sequencing or selections. This limits the application scope of the method.

Gene Reassembly

Method description: Gene variants are digested using restriction enzymes and relegated to form hybrid genes encoding hybrid enzymes.

Example: A library of >20,000 hybrid genes was generated by gene reassembly of three α -amylase wild-type genes that encoded enzymes with varying properties. Improved mutants with combined optimal phenotypes of expression, temperature stability, and pH optimum were obtained [34].

Note: The rational design element as well as the randomness of the religation procedure limits the utility of these libraries, as additional genetic engineering to create and or remove restriction sites may often be required and a large number of variants need to be screened.

Assembly of Designed Oligonucleotides (ADO)

Method description: A synthetic shuffling approach based on oligonucleotides whose design is guided by sequence information.

Example: From a library prepared with information from two *B. subtilis* lipase genes, several variants were obtained that displayed increased enantioselectivity in a model reaction involving the hydrolysis of a *meso*-diacetate [35].

Note: A synthetic shuffling approach.

Biased Mutation Assembling

Method description: The library construction process is manipulated by providing different fragments, e.g., wild-type mutant, in different ratios to favor only a few or multiple mutations in the progeny.

Example: The method was applied to the generation of more thermostable prolyl endopeptidases. A mutant with a 1200-fold longer activity, half-life at 60°C was identified. [36].

NExT DNA Shuffling

Method description: Uridine triphosphate (dUTP) is used during the amplification of a gene library by PCR. The incorporated uracil bases were excised using uracil-DNA-glycosylase and the DNA backbone subsequently cleaved with piperidine. Polyacrylamide urea gels demonstrated adjustable fragmentation size over a wide range. The oligonucleotide pool was reassembled by internal primer extension to full length with a proofreading polymerase to improve yield over Taq.

Example: Chloramphenicol acetyltransferase was shuffled using a 33% dUTP PCR, which resulted in shuffled clones (average fragment size of 86 bases) and revealed a low mutation rate (0.1%) [37].

Note: An alternative to Miyazaki's method [31].

Multiplex-PCR-Based Recombination (MUPREC)

Method description: A method for the *in vitro* recombination of single-point mutations that reduces the introduction of novel point mutations, which usually occur during recombination processes. A multiplex-PCR reaction generates gene fragments that contain preformed point mutations. These fragments are subsequently assembled into full-length genes by a recombination-PCR step. The process of MUPREC does not require DNase I digestion for gene-fragmentation. The low error rate resulted in high-quality variant libraries of true recombinants, thereby minimizing the screening efforts and saving time and money.

Example: The MUPREC method was used in the directed evolution of a *B. subtilis* lipase that can catalyze the enantioselective hydrolysis of a model *meso*-compound. The method was useful in producing a reliable second-generation library of true recombinants from which better performing variants were identified by using a high-throughput electrospray ionization mass spectrometry (ESI-MS) screening system [38].

Note: A method to reduce random mutagenesis during the recombination procedure.

Offset Recombinant PCR (OR-PCR)

Method description: A method that uses standard PCR to promote high recombination frequencies among compact heterologous domains.

Example: Pfu polymerase generated chimeric crossover events in 13% of the population when markers were separated by only 70 nt. The fraction of recombinant sequences reached 42% after six consecutive rounds of PCR, a value close to 50% expected from a fully shuffled population [39].

Note: This study describes how variations in the PCR process can influence the library quality.

30.4.2.3.2 Family Shuffling (Figure 30.6)

Method description: Libraries of hybrid genes were generated by random fragmentation of a pool of homologous genes, and reassembly of the fragments in a self-priming polymerase reaction.

Example: Moxalactamase activity was evolved when four different cephalosporinase genes were shuffled together instead of separately. The best clone was up to 540-fold improved

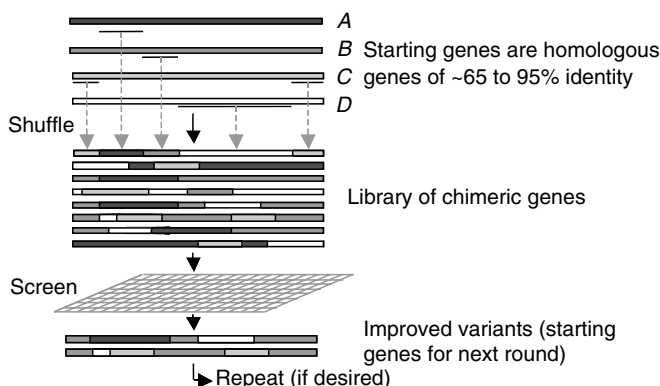


FIGURE 30.6 (See color insert following page 526) Family shuffling. Various genes encoding enzymes with identical (or at least similar) function are recombined to provide libraries of chimeric genes. The diversity introduced in this method is functional since it has been preselected in nature. In addition to full-length genes, partial genes and oligonucleotides can be added to the library generation procedure, thereby improving the quality of the libraries that are generated.

and contained eight segments from three of the four genes as well as 33 amino-acid mutations [40].

Note: Homologous genes provide functional diversity that has been tested in nature, in contrast to random diversity that is generated in the laboratory and that has not been subjected to selection mechanisms.

Single-Stranded DNA Shuffling

Method description: Family shuffling efficiency is reportedly improved by using complementary strands of the two parents.

Example: ssDNAs of two catechol 2,3-dioxygenase genes were fragmented and reassembled. The frequency of obtaining hybrid genes was 14-fold higher compared to double stranded family shuffling and more thermostable enzyme variants were obtained [41].

Note: This method may provide advantages only when two genes are shuffled; with an increased number of parents some bias may slip in.

Combinatorial Libraries Enhanced by Recombination in Yeast (CLERY)

Method description: A family shuffling strategy involving both PCR-based and *in vivo* recombination and expression in yeast.

Example: Two human cytochrome P450s were shuffled and screened in yeast. Sequence analysis of randomly picked and functionally selected clones confirmed the shuffling efficiency [42].

Note: A method that should be useful for the evolution of enzymes that are difficult to express in bacteria, but not in eukaryotic systems like yeast.

Degenerate Oligonucleotide Gene Shuffling (DOGS)

Method description: A shuffling method in which degenerate primers control the relative levels of recombination between the parents thereby increasing the recombination frequency.

Example: A diverse family of β -xylanase genes that differ widely in G + C content was shuffled [43].

Note: This procedure avoids the use of endonucleases for gene fragmentation prior to shuffling and allows the use of random mutagenesis of selected segments of the gene as part of the procedure.

Rachitt

Method description: Randomly cleaved parental DNA fragments are annealed to a transient polynucleotide scaffold resulting in chimeric libraries that average a high number of crossovers per gene.

Example: A monooxygenase was evolved for increased rate and extent of biodesulfurization on complex substrates, as well as for 20-fold faster conversion of a nonnatural substrate [44].

Note: A laborious procedure that provides many crossovers between genes.

Degenerate Homoduplex Gene Family Recombination (DHR)

Method description: A synthetic DNA recombination method that provides more random recombination

Example: A chimeric protein whose agonist activity was enhanced 123-fold was obtained from a human–mouse chimeric epidermal growth factor library [45].

Note: The authors suggest that this is a less-biased approach to DNA shuffling that should be useful for engineering of a wide variety of proteins.

30.4.2.3.3 “Distant” Family Shuffling

Incremental Truncation for the Creation of Hybrid Enzymes (ITCHY)

Method description: ITCHY creates combinatorial fusion libraries between genes independent of DNA homology.

Example: Fragments of the *E. coli* and human glycinamide ribonucleotide transformylase genes (50% identity at the DNA level) were fused by ITCHY and DNA shuffling. ITCHY identified a more diverse set of active fusion points including those in regions of nonhomology [46].

Note: The number of active clones in such libraries is small and very high-throughput or selection methods are needed to identify active variants.

SCRATCHY

Method description: The approach combines ITCHY and DNA shuffling. A library of hybrid enzymes created by ITCHY is subjected to a DNA-shuffling step to augment the number of crossovers.

Example: Functional hybrid enzymes containing multiple crossovers were obtained from SCRATCHY libraries that were created from the glycinamide-ribonucleotide formyltransferase genes from *E. coli* and human [47].

Sequence Homology-Independent Protein Recombination (SHIPREC)

Method description: A method to create single crossover libraries of distant (unrelated) genes at structurally related sites.

Example: A library of a human membrane-associated cytochrome P450 and the bacterial heme domain of a soluble P450 was generated and properly folded variants were selected with an antibiotic marker. Screening for the activity of the human enzyme identified two functional P450 hybrids with improved expression in *E. coli* [48].

Note: A method for chimera genesis of unrelated enzymes with an interesting application.

Structure-Based Combinatorial Protein Engineering (SCOPE)

Method description: A semirational protein engineering approach that uses information from protein structure coupled with established DNA manipulation techniques to design and create multiple crossover libraries from nonhomologous genes.

Example: Libraries of chimeric genes of a rat and a African swine fever virus DNA polymerase with up to five crossovers were synthesized in a series of PCR reactions by employing hybrid oligonucleotides that code for variable connections between structural elements. Genetic complementation in *E. coli* enabled identification of several novel DNA polymerases with enhanced phenotypes [49].

Note: Rational protein engineering coupled with a recombination approach increases the functional diversity in the generated libraries.

Enhanced Crossover SCRATCHY

Method description: Gene variants prepared by ITCHY technology are shuffled so that multiple crossover points can be found in the progeny of the evolved library.

Example: Libraries were generated from rat and human glutathione transferase genes, while random sequencing indicated greater diversity than obtained by family shuffling or SCRATCHY itself. Several variants were isolated by HTP flow-cytometry that had retained rat-like substrate specificity [50].

Note: The best variants were primarily rat sequence with the only human DNA found at the 3'-end of the gene. The technology demonstrates that dramatic changes in the primary structure often result in loss of function.

Sequence-Independent Site-Directed Chimeragenesis (SISDC)

Method description: A computer-aided method using the SCHEMA algorithm [51] for the facile recombination of distantly related (or unrelated) genes at multiple discrete sites using endonucleases.

Example: Two β -lactamases were recombined at seven sites and screened for functionality. Among the active clones, 14 unique chimeras were identified [52].

Note: The use of computational methods increases the probability of generating hybrid enzymes with general activity.

30.4.2.3.4 Pathway Shuffling

Method description: DNA shuffling was used for the evolution of an operon.

Example: A three-gene arsenate resistance operon encoding two enzymes and a transcriptional regulator was evolved by DNA shuffling, involving three rounds of *in vitro* recombination, followed by selection in *E. coli* for increased resistance. The evolved cells grew in up to 0.5 M arsenate, a 40-fold increase in resistance. The resulting shuffled operon had spontaneously integrated in the chromosome and contained 13 mutations [53].

Note: These results demonstrate that the fitness of proteins and cells is determined by many parameters some of which are poorly understood.

30.4.2.3.5 Genome Shuffling

Method description: Whole-genome shuffling is an *in vivo* procedure that combines the advantage of multiparental crossing allowed by DNA shuffling with the recombination of entire genomes normally associated with conventional breeding. Recursive genomic recombination within a population of bacteria efficiently generated combinatorial libraries of new strains.

Example: Production of tylosin in *Streptomyces fradiae* was improved several fold [54].

Note: This approach has the potential to facilitate cell and metabolic engineering and provides a nonrecombinant alternative to the rapid generation of improved organisms.

30.5 EVOLVING ENZYMES FOR THE PROCESS

Given the potential utility of enzymes for the synthesis of complex chiral molecules and the sensitivity of these biocatalysts under typical chemical processing conditions, it is understandable that one of the most active applications of directed evolution has been for the use of enzymes in chemical synthesis. [Section 30.4](#) provided an overview of laboratory methods that were developed over the last decade for the generation of large enzyme libraries from which improved enzymes may be isolated. The applicability of natural enzymes was recently summarized as “Natural selection has created a large variety of enzymes superbly adapted to catalyze an array of chemical reactions. However, there are still too few enzymes available to catalyzing reactions of interest and many of these have suboptimal properties for processing conditions.” [55]. Before, chemical processes were developed to optimally utilize the potential performance of the biocatalyst, optimal chemical processes can now be designed from first principles, followed by the development of the biocatalyst to enable the designed process ([Figure 30.7](#)).

A robust manufacturing process is characterized by commercially important constraints such as:

- High volumetric productivity with at least 10% substrate.
- Chirality introduced early in the process by chiral synthesis or resolution using cheap reagents.
- Raw materials that are commercially available in large scale, rather than through lab suppliers.
- Inexpensive solvents rather than expensive aprotic solvents.
- Reaction work-ups that are simple, involving filtration, extraction and distillation, not evaporation to dryness, drying over magnesium sulfate, or chromatography.

Generally, these constraints are not an issue for biocatalytic processes, as enzymes have exquisite enantioselectivity (or it can be evolved into the enzyme), are chemoselective, circumventing the need for complicated separation processes in work-up, and can run with high

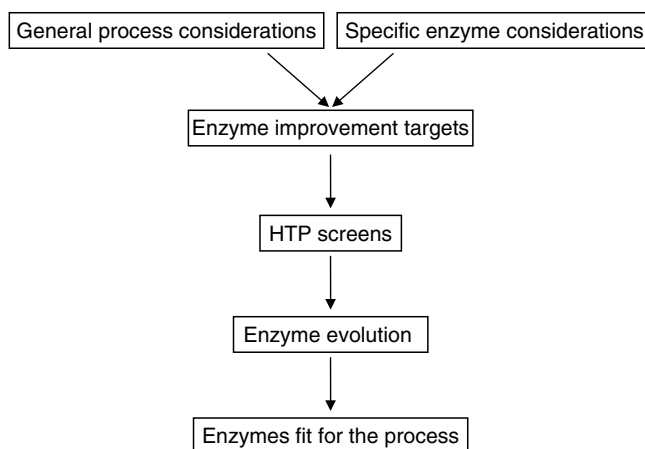


FIGURE 30.7 Generating the optimal enzyme for the process of interest. Before embarking on the enzyme improvement process, the process of interest is designed and process parameters are defined. An appropriate starting enzyme is identified and evaluated under the process conditions of choice to provide a set of enzyme improvement targets. Based on these targets, HTP screens are designed and developed so that enzyme evolution can be initiated. Iterative rounds of evolution will provide the enzyme of interest.

productivity. The extent to which all criteria can be met varies on an enzyme-by-enzyme and process-by-process basis. Enzyme manufacturing is a well-established technology that is available to (almost) every enzyme of interest and as a result, enzyme cost is predictable. Using more active variants and thereby decreasing enzyme loading and increasing the substrate-to-catalyst ratio can further lower the enzyme cost contribution to a process. Finally, expensive cosubstrates can be reused using efficient regeneration systems.

Based on these general guidelines and the known characteristics of the enzyme, a process is designed and the typically desired biocatalyst improvements are:

- Higher specific activity
- Increased or maintained enantioselectivity
- Increased solvent tolerance
- Operable at lower cofactor concentrations
- Increased in-process stability
- Reduced inhibition by substrate and product

The parameters that are determined to be key for the desired catalyst are then used to develop the tiered screening approach, addressing simultaneously as many of these parameters as possible in the earliest possible tier. Only in this way can the number of assumptions that have to be made when downscaling a chemical process to a HTP format, be kept at a minimum.

When this approach is well executed, enzymes will emerge that catalyze the desired conversion with a high volumetric productivity (high specific activity, stability, not inhibited) to give high quality product (purity and enantiomeric excess). The small amount of enzyme that is needed for these reactions simplifies the handling of the catalyst and facilitates the work-up of the reaction. Since the enzyme is biological in nature, it can easily be disposed of with the normal waste-treatment stream and no precautions are required in ensuring the absence of toxic heavy metals in the waste stream or in the product of the reaction.

30.6 APPLICATIONS OF DIRECTED EVOLUTION FOR BIOCATALYSIS

Evolution of enzymes for biocatalysis, that is, the preparation of chemicals using enzymes outside of a living cell, has been one of the most important applications of directed evolution technologies. While, it has been recognized for more than a century that the application of enzymes to organic synthesis is highly attractive [56], practical application of these biocatalysts has been limited to a large degree by the limitations of wild-type proteins evolved to function within a living cell. Two primary application areas that have seen recent success are the increase in activity and stability of enzymes in the presence of high concentrations of organic substrates (and thus increase of volumetric productivity) and the evolution of enantioselective enzymes.

30.6.1 ORGANIC SOLVENT STABILITY

Drug molecules and intermediates are often hydrophobic in nature and so, high activity and stability in the presence of high concentrations of organic substrates and solvent is often desirable. Virtually all enzymes evolved in nature to be active and stable in water. Zaks and Klivanov showed that while some lipases can be stable in very hydrophobic media, the activity of most enzymes tends to be orders of magnitude lower in nonnatural media than in aqueous media [57]. Thus, it is surprising that relatively few evolution studies have been directed toward producing biocatalysts that are tolerant of high concentrations of organic solvents and hydrophobic substrates. Woodley has pointed out that while one would like to evolve enzymes for activity and stability in the presence of organic substrates and cosolvent, there is a bias toward performing directed evolution screening in growth media under conditions that are amenable to cell growth and protein production [58]. Growth conditions are unlikely to accurately mimic the environment that the biocatalyst would experience in an industrial setting. Separation of enzyme production from fitness of function testing, as in the examples given below, has allowed evolution of activity and stability toward organic media.

30.6.2 ENANTIOSELECTIVITY AND ENANTIOSPECIFICITY

Enantiopurity, the single most important driver for developing a biocatalytic process, is often one of the most difficult parameters to improve by directed evolution methods due to the paucity of HTP methods for determination of enantiomeric purity. Frequently, the enantioselectivity and enantiospecificity of a wild-type enzyme for an unnatural substrate is low and cannot be improved by reaction engineering. A rational and systematic method to evolve enantiospecificity and enantioselectivity in enzymes will transform biocatalysis by imparting the ability to adapt enzymes to fit the target substrate and process [59,60]. Directed evolution by DNA shuffling, which does not require extensive screening of libraries appears to lend itself to complex problems, such as increasing enantioselectivity. A complementary method to limit library size is to use either a protein structural model of the parent enzyme to target regions in the protein, which may have a higher probability of impacting chiral interactions, or the use of mutant library generation in which a small sampling of the library gives an indication of overall library content.

30.6.3 EXAMPLES OF EVOLVED ENZYMES FOR BIOCATALYSIS APPLICATIONS

30.6.3.1 Amino Acid Oxidase

The dynamic kinetic “deracemization” of chiral amines was enabled by evolving an enantioselective amino acid oxidase with broad substrate specificity that is coupled with a nonselective

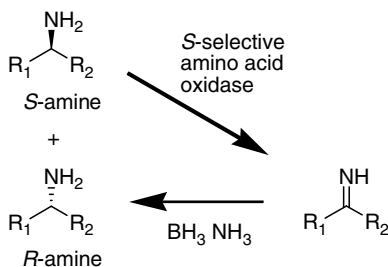


FIGURE 30.8 Amine “deracemization” through *S*-selective amino acid oxidase (R_1 : alkyl, aryl; R_2 : methyl).

imine reduction [61,62] (Figure 30.8). The enantioselective oxidase preferentially converts one enantiomer of the amine to an imine, (typically the *S*-amine), and then the nonspecific chemical reductant converts the imine back to the *R,S*-amine resulting in enrichment in the unoxidized (*R*)-amine. The process is run until the *R,S*-mixture is converted to the single enantiomer in high optical purity. Error-prone PCR was used to generate mutants of an *Aspergillus niger* derived amino acid oxidase. A colorimetric screen based on the generation of hydrogen peroxide enabled evaluation of ~150,000 clones for enantioselectivity in the oxidation of α -benzylamine. A single Asn336Ser mutation imparted a 47-fold increase in activity and a fivefold increase in enantioselectivity on this substrate. Surprisingly, the single mutant showed greatly improved selectivity and activity on a wide range of substrates. The results are unusual when compared with other directed evolution studies in that a single mutation resulted in not only an increase of activity and enantioselectivity, but also a significant broadening of substrate specificity.

30.6.3.2 Hydantoinase

One of the first demonstrations of the plasticity of enzymes for modification of enantiospecificity was the inversion of specificity of a hydantoinase for the production of *L*-methionine [63]. Inversion of enantiopreference from *D* to modest *L* specificity gave rise to increased throughput of a dynamic kinetic resolution system based on a racemase, hydantoinase, and *L*-carbamoylase combination. As outlined in Figure 30.9, the optical purity of *L*-methionine is determined by the specificity of the carbomoylase, but increasing the *L*-preference of the hydantoinase increases throughput by increasing the concentration of the *L*-carbamoyl methionine precursor rather than the nonproductive *D*-carbamoyl pathway. Following a now common strategy, an initial round of random mutagenesis was performed to identify sensitive residues, followed by saturation mutagenesis to screen all possible amino acid changes at these sensitive positions. Interestingly on scale-up the inverted selectivity found in high-throughput screening was only evident in the presence of the expression media component present in screening. A single amino acid change I95F was responsible for the inversion of selectivity. Subsequent coexpression of the three-enzyme system: the racemase, the moderately *L*-selective hydantoinase and the *L*-carbamoylase led to reaching 90% conversion five times faster than the parent hydantoinase system. The study indicates a number of important features common to directed evolution programs, that is, the sensitivity to screening conditions (“you get what you screen for”), the ability of proteins to make large changes in function with apparently minor amino acid substitutions, and the difficulty in predicting such changes *a priori*.

30.6.3.3 Epoxide Hydrolase

More recently, Janssen et al. evolved an epoxide hydrolase for the hydrolytic kinetic resolution of styrene epoxide (Figure 30.10) [64]. Analogous to the hydantoinase program,

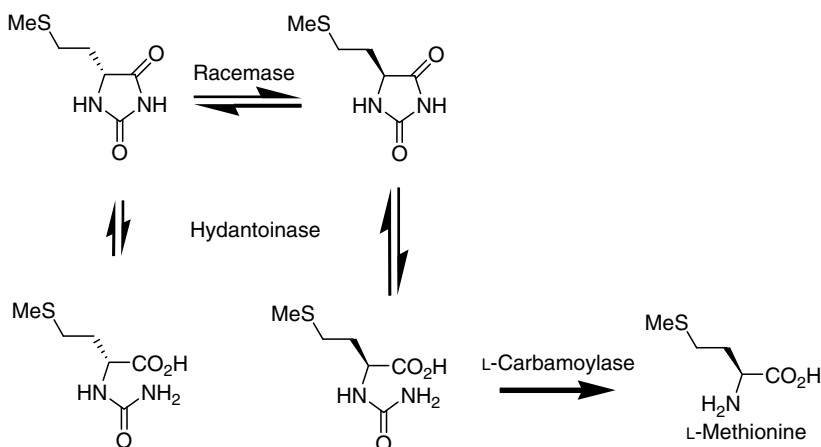


FIGURE 30.9 Dynamic kinetic resolution for the synthesis of L-Methionine.

a first round of screening of 40,000 clones from error-prone PCR with two to three mutations per mutant was used to identify mutants with improved enantiospecificity. Of these, 15 were selected for gene shuffling [12] to recombine beneficial mutations. A library of 20,000 clones was screened for activity in an agar plate assay and of these, eight variants were identified with E values ranging from 20 to 44 (vs. 3.4 for the WT) on p -nitrophenyl glycidyl ether (NPGE). The most selective variants contained three or more mutations, including at least one change in an active site tyrosine. Interestingly, kinetic characterization showed that the source of improved enantiospecificity on NPGE in the protein differed for different mutants. Mutations that were responsible for increased specificity over the wild type gave rise to increased differentiation via either changes in the relative K_m or k_{cat} for the two enantiomers. Screening of the eight clones with high specificity on NPGE on other epoxides showed that each had increased selectivity on at least three additional epoxides.

30.6.3.4 Lipase

Reetz et al. [65] demonstrated the utility of a “focused directed evolution approach” in an enzyme model by targeting amino acid residues near the active site, which are likely to interact with the substrate to broaden the substrate selectivity of a lipase. Randomization of residues in the substrate-binding pocket of *Pseudomonas aeruginosa* lipase resulted in the acceptance of α -substituted carboxylic acid esters by this enzyme. While α -methyl branching gave low rates of reaction with p -nitrophenyl esters with the natural enzyme, aryl substitution at the alpha position was tolerated with moderate enantiospecificity in a single and double mutant. It was the supposition that pairs of amino acid changes would be required; however, single mutations were found to impart the broadest substrate acceptance and moderate selectivity ($E = 20$ –25) on bulky esters that were not accepted by the wild-type lipase. Broadening the

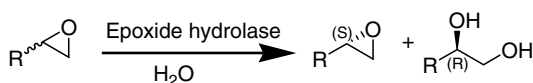


FIGURE 30.10 Hydrolytic resolution of epoxides (R = aryl, chloroethyl, butyl).

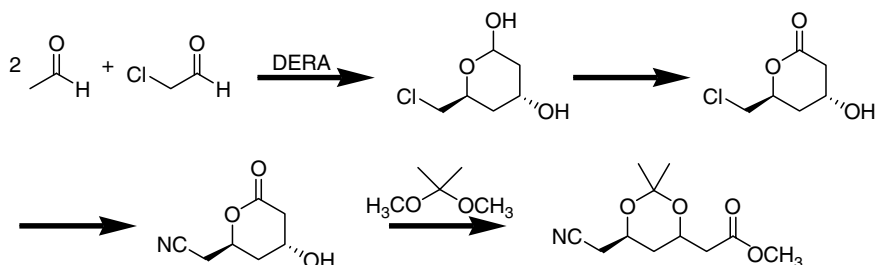


FIGURE 30.11 Potential route to key atorvastatin intermediate through aldolase coupling.

substrate range to bulkier side chains was not simply a result of decreasing steric bulk of residues in the active site, since L17F mutation resulted in some of the widest substrate tolerance.

30.6.3.5 Aldolase

In the evolution of a 2-deoxy-D-ribosephosphate aldolases (DERA) for construction of statin side chains, stability in the presence of high concentrations of the reactive substrates acetaldehyde and chloroacetaldehyde was necessary to make the process viable [66]. The installation of both chiral centers by DERA in an intermediate for the HMG CoA reductase inhibitor atorvastatin (Lipitor) is shown in Figure 30.11. Two rounds of random mutagenesis were performed on the aldolase to select for more stable mutants, followed by recombination of stability and activity hits. A double mutant with tenfold improved yield was identified. The route is attractive in that chirality was installed with excellent atom economy using relatively inexpensive reagents, although Mink notes that the reaction is made complex by the high reactivity of these aldehydes.

30.6.3.6 Nitrilase

Evolution of a biocatalyst for use in a potential industrial process often requires the evolution of several parameters at once. Desantis et al. [67] reported the directed evolution of a nitrilase for the synthesis of a chiral intermediate for atorvastatin from prochiral 3-hydroxyglutaronitrile (Figure 30.12). A screen of 200 nitrilases identified a wild-type enzyme that effectively carried out this reaction at an industrially relevant substrate concentration (3 M). However, the enantiomeric excess of the (*R*)-4-cyano-3-hydroxybutyric acid product was only 87.6%. Gene site saturation mutagenesis was used to generate libraries of each of the 20 amino acids at every residue of the enzyme. Using HTP mass spectrometry assay with ^{15}N -labeled *R*-dinitrile, >30,000 clones were evaluated. The libraries were screened for substrate tolerance, productivity, and enantioselectivity and the best variant was found to contain an Ala190His mutation that gave a product with 98.5% ee at 3 M substrate loading and with an overall volumetric productivity of 619 g/L/d. At first glance, this process appears to be extremely simple: a single enzymatic transformation on an inexpensive prochiral dinitrile. However, the process does not give the desired ester but a hydroxy acid. Thus, precipitation of the half-acid (e.g., calcium



FIGURE 30.12 Nitrilase desymmetrization for hydroxynitrile acid.

30.6.3.7 Ketoreductase, Glucose Dehydrogenase, and Halohydrin Dehalogenase

Step 1

ClCC(=O)CC(=O)OCC $\xrightarrow[\beta\text{-NADP}^+]{\text{Ketoreductase}, \beta\text{-NADPH} + \text{H}^+}$ ClC[C@H](O)CC(=O)OCC

Step 2

ClC[C@H](O)CC(=O)OCC $\xrightarrow[\text{NaCN}]{\text{Halohydrin dehalogenase}}$ NC[C@H](O)CC(=O)OCC + NaCl

The diagram illustrates a biocatalytic process for the synthesis of "Hydroxynitrile". The process begins with a biocatalytic reduction step where ethyl 3-chloropentanoate (CCCC(=O)OCC) is reduced to ethyl 3-hydroxypentanoate (CCCC(O)C(=O)OCC) using NaOH (aq.) and a biocatalytic system containing glucose, KRED cofactor, and GDH. The reaction mixture is then filtered. The filtrate is extracted with solvent, which is recycled. The aqueous phase is then subjected to biocatalytic cyanation using HHDH and HCN. The resulting mixture is again extracted with solvent, which is recycled. The aqueous phase is then subjected to short-path distillation to yield the final product, "Hydroxynitrile" (CCCC(O)C#N). The process also includes a step for HCN to bleach treatment and a final filtration step before the product is collected.

© 2006 by Taylor & Francis Group, LLC.

cyanation using a halohydrin dehalogenase evolved to efficiently catalyze the displacement of chloride by cyanide. A combination of several directed evolution technologies including error-prone PCR, site directed mutagenesis, focused evolution, NNN randomization, semi-synthetic shuffling, and family shuffling was used to increase the volumetric productivity of the halohydrin dehalogenase more than 4000-fold by mutation of ~15% of the amino acids of the protein, including 4 of the 11 residues indicated to be directly in the active site [70]. The overall process is operationally very simple with direct extraction of the intermediate and product from the reaction(Figure 30.14).

30.7 CONCLUSION AND PROGNOSIS

The confluence of advancements in HTP screening and recombination technologies, and the rapid expansion of available genetic diversity has led to a renaissance in biocatalysis. For decades, the potential of biocatalysis for synthesis of chiral and complex molecules was great, but was largely limited by the inefficiency of these catalysts on unnatural substrates and sensitivity to typical processing conditions in a chemical plant. Examples described here provide proof that directed evolution of enzymes for chemical production is a powerful option for creating highly efficient processes that exceed the performance of processes based on traditional chemical catalysis. The malleability not only of catalytic efficiency and (stereo- and chemo-) selectivity, but of catalytic function as well, indicates that we are just beginning to explore the potential of creating new biocatalytic activities.

REFERENCES

1. Magnusson, L.U., Farewell, A., and Nystrom, T., ppGpp: a global regulator in *Escherichia coli*, *Trends Microbiol.*, 13, 236–242, 2005.
2. Porankiewicz, J., Wang, J., and Clarke, A.K., New insights into the ATP-dependent Clp protease: *Escherichia coli* and beyond, *Mol. Microbiol.*, 32, 449–458, 1999.
3. Bochtler, M., Ditzel, L., Groll, M., et al., The proteasome, *Annu. Rev. Biophys. Biomol. Struct.*, 28, 295–317, 1999.
4. Gonzales, T. and Robert-Baudouy, J., Bacterial aminopeptidases: properties and functions, *FEMS Microbiol. Rev.*, 18, 319–344, 1996.
5. Carrard, G., Bulteau, A.L., Petropoulos, I., et al., Impairment of proteasome structure and function in aging, *Int. J. Biochem. Cell. Biol.*, 34, 1461–1474, 2002.
6. Dice, J.F. and Goldberg, A.L., A statistical analysis of the relationship between degradative rates and molecular weight of proteins, *Arch. Biochem. Biophys.*, 170, 213–319, 1975.
7. Choe, J.Y., Nelson, S.W., Arienti, K.L., et al., Inhibition of fructose-1,6-bisphosphatase by a new class of allosteric effectors, *J. Biol. Chem.*, 278, 51176–51183, 2003.
8. Fenton, A.W., and Reinhart, G.D., Mechanism of substrate inhibition in *Escherichia coli* phospho-fructokinase, *Biochemistry*, 42, 12676–12681, 2003.
9. Chassagnole, C., Noisommit-Rizzi, N., Schmid, J.W., et al., Dynamic modeling of the central carbon metabolism of *Escherichia coli*, *Biotechnol. Bioeng.*, 79, 53–73, 2002.
10. Baik, S.H., Ide, T., Yoshida, H., et al., Significantly enhanced stability of glucose dehydrogenase by directed evolution, *Appl. Microbiol. Biotechnol.*, 61, 329–335, 2003.
11. Ashina, Y. and Suto, M., Development of an enzymatic process for manufacturing acrylamide and recent progress, *Bioprocess Technol.*, 16, 91–107, 1993.
12. Stemmer, W.P., Rapid evolution of a protein *in vitro* by DNA shuffling, *Nature*, 370, 389–391, 1994.
13. Zhang, N., Stewart, B.G., Moore, J.C., et al., Directed evolution of toluene dioxygenase from *Pseudomonas putida* for improved selectivity toward *cis*-indandiol during indene bioconversion, *Metab. Eng.*, 2, 339–348, 2000.
14. Entrez Genome Project. Available at: <http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>.

15. Murakami, H., Hohsaka, T., and Sisido, M., Random insertion and deletion of arbitrary number of bases for codon-based random mutation of DNAs, *Nat. Biotechnol.*, 20, 76–81, 2002.
16. Pikkemaat, M.G. and Janssen, D.B., Generating segmental mutations in haloalkane dehalogenase: a novel part in the directed evolution toolbox, *Nucl. Acids Res.*, 30, e3s–5, 2002.
17. Wong, T.S., Tee, K.L., Hauer, B., et al., Sequence saturation mutagenesis (SeSaM): a novel method for directed evolution, *Nucl. Acids Res.*, 32, e26, 2004.
18. Fox, R., Roy, A., Govindarajan, S., et al., Optimizing the search algorithm for protein engineering by directed evolution, *Protein Eng.*, 16, 589–597, 2003.
19. Fox, R., Directed molecular evolution by machine learning and the influence of nonlinear interactions, *J. Theor. Biol.*, 234, 187–199, 2005.
20. Otten, L.G. and Quax, W.J., Directed evolution: selecting today's biocatalyst, *Biomol. Eng.*, 22, 1–9, 2005.
21. Roodveldt, C., Aharoni, A., and Tawfik, D.S., Directed evolution of proteins for heterologous expression and stability, *Curr. Opin. Struct. Biol.*, 15, 50–56, 2005.
22. Lutz, S. and Patrick, W.M., Novel methods for directed evolution of enzymes: quality, not quantity, *Curr. Opin. Biotechnol.*, 15, 291–297, 2004.
23. Neylon, C., Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution, *Nucl. Acids Res.*, 32, 1448–1459, 2004.
24. Stemmer, W.P., DNA shuffling by random fragmentation and reassembly: *in vitro* recombination for molecular evolution, *Proc. Natl. Acad. Sci. USA*, 91, 10747–10751, 1994.
25. Zhao, H. and Arnold, F.H., Optimization of DNA shuffling for high fidelity recombination, *Nucl. Acids Res.*, 25, 1307–1308, 1997.
26. Zhao, H., Giver, L., Shao, Z., et al., Molecular evolution by staggered extension process (StEP) *in vitro* recombination, *Nat. Biotechnol.*, 16, 258–261, 1998.
27. Volkov, A.A., Shao, Z., and Arnold, F.H., Recombination and chimeragenesis by *in vitro* heteroduplex formation and *in vivo* repair, *Nucl. Acids Res.*, 27, e18, 1999.
28. Melnikov, A. and Youngman, P.J., Random mutagenesis by recombinational capture of PCR products in *Bacillus subtilis* and *Acinetobacter calcoaceticus*, *Nucl. Acids Res.*, 27, 1056–1062, 1999.
29. Kim, G.J., Cheon, Y.H., Park, M.S., et al., Generation of protein lineages with new sequence spaces by functional salvage screen, *Protein Eng.*, 14, 647–654, 2001.
30. Ness, J.E., Kim, S., Gottman, A., et al., Synthetic shuffling expands functional protein diversity by allowing amino acids to recombine independently, *Nat. Biotechnol.*, 20, 1251–1255, 2002.
31. Miyazaki, K., Random DNA fragmentation with endonuclease V: application to DNA shuffling, *Nucl. Acids Res.*, 30(24), e139, 2002.
32. Song, J.K., Chung, B., Oh, Y.H., et al., Construction of DNA-shuffled and incrementally truncated libraries by a mutagenic and unidirectional reassembly method: changing from a substrate specificity of phospholipase to that of lipase, *Appl. Environ. Microbiol.*, 68, 6146–6151, 2002.
33. Hayes, R.J., Bentzien, J., Ary, M.L., et al., Combining computational and experimental screening for rapid optimization of protein properties, *Proc. Natl. Acad. Sci. USA*, 99, 15926–15931, 2002.
34. Richardson, T.H., Tan, X., Frey, G., et al., A novel, high performance enzyme for starch liquefaction. Discovery and optimization of a low pH, thermostable alpha-amylase, *J. Biol. Chem.*, 277, 26501–26507, 2002.
35. Zha, D., Eipper, A., and Reetz, M.T., Assembly of designed oligonucleotides as an efficient method for gene recombination: a new tool in directed evolution, *ChemBiochem*, 4, 34–39, 2003.
36. Hamamatsu, N., Aita, T., Nomiya, Y., et al., Biased mutation-assembling: an efficient method for rapid directed evolution through simultaneous mutation accumulation, *Protein Eng. Des. Sel.*, 18, 265–271, 2005.
37. Muller, K.M., Stebel, S.C., Knall, S., et al., Nucleotide exchange and excision technology (NExT) DNA shuffling: a robust method for DNA fragmentation and directed evolution, *Nucl. Acids Res.*, 33, e117, 2005.
38. Eggert, T., Funke, S.A., Rao, N.M., et al., Multiplex-PCR-based recombination as a novel high-fidelity method for directed evolution, *ChemBiochem*, 6, 1062–1067, 2005.

39. Rozak, D.A. and Bryan, P.N., Offset recombinant PCR: a simple but effective method for shuffling compact heterologous domains, *Nucl. Acids Res.*, 33(9), e82, 2005.
40. Cramer, A., Raillard, S.A., Bermudez, E., et al., DNA shuffling of a family of genes from diverse species accelerates directed evolution, *Nature*, 391, 288–291, 1998.
41. Kikuchi, M., Ohnishi, K., and Harayama, S., An effective family shuffling method using single-stranded DNA, *Gene*, 243, 133–137, 2000.
42. Abecassis, V., Pompon, D., and Truan, G., High efficiency family shuffling based on multi-step PCR and *in vivo* DNA recombination in yeast: statistical and functional analysis of a combinatorial library between human cytochrome P450 1A1 and 1A2, *Nucl. Acids Res.*, 28, E88, 2000.
43. Gibbs, M.D., Nevalainen, K.M., and Bergquist, P.L., Degenerate oligonucleotide gene shuffling (DOGS): a method for enhancing the frequency of recombination with family shuffling, *Gene*, 271, 13–20, 2001.
44. Coco, W.M., Levinson, W.E., Crist, M.J., et al., DNA shuffling method for generating highly recombined genes and evolved enzymes, *Nat. Biotechnol.*, 19, 354–359, 2001.
45. Coco, W.M., Encell, L.P., Levinson, W.E., et al., Growth factor engineering by degenerate homoduplex gene family recombination, *Nat. Biotechnol.*, 20, 1246–1250, 2002.
46. Ostermeier, M., Shim, J.H., and Benkovic, S.J., A combinatorial approach to hybrid enzymes independent of DNA homology, *Nat. Biotechnol.*, 17, 1205–1209, 1999.
47. Lutz, S., Ostermeier, M., Moore, G.L., et al., Creating multiple-crossover DNA libraries independent of sequence identity, *Proc. Natl. Acad. Sci. USA*, 98, 11248–11253, 2001.
48. Sieber, V., Martinez, C.A., and Arnold, F.H., Libraries of hybrid proteins from distantly related sequences, *Nat. Biotechnol.*, 19, 456–460, 2001.
49. O'Maille, P.E., Bakhtina, M., and Tsai, M.D., Structure-based combinatorial protein engineering, *J. Mol. Biol.*, 321, 677–691, 2002.
50. Kawarasaki, Y., Griswold, K.E., Stevenson, J.D., et al., Enhanced crossover SCRATCHY: construction and high-throughput screening of a combinatorial library containing multiple non-homologous crossovers, *Nucl. Acids Res.*, 31, e126, 2003.
51. Voight, C.A., Martinez, C., Wang, Z.G., et al., Protein building blocks preserved by recombination, *Nat. Struct. Biol.*, 9, 553–558, 2002.
52. Hiraga, K. and Arnold, F.H., General method for sequence-independent site-directed chimeragenesis, *J. Mol. Biol.*, 330, 287–296, 2003.
53. Cramer, A., Dawes, G., Rodriguez, E., Jr., et al., Molecular evolution of an arsenate detoxification pathway by DNA shuffling, *Nat. Biotechnol.*, 15, 436–438, 1997.
54. Zhang, Y.X., Perry, K., Vinci, V.A., et al., Genome shuffling leads to rapid phenotypic improvement in bacteria, *Nature*, 415, 644–646, 2002.
55. Bommarius, A. and Bommarius-Riebel, B., *Biocatalysis: Fundamentals and Applications*, John Wiley, 2005.
56. Halling, P. and Kvittingen, L., Why did biocatalysis in organic media not take off in the 1930s? *Trends Biotechnol.*, 17, 334–344, 1999.
57. Zaks, A. and Klivanov, A., Enzymatic catalysis in organic media at 100 degrees C, *Science*, 224, 1249–1251, 1994.
58. Lye, G.J., Dalby, P.A., and Woodley, J.M., Better biocatalytic processes faster: new tools for the implementation of biocatalysis in organic synthesis, *Org. Proc. Res. Dev.*, 6, 434–440, 2002.
59. Reetz, M., Changing the enantioselectivity of enzymes by directed evolution, *Methods Enzymol.*, 388, 238–256, 2004.
60. Turner, N.J., Directed evolution of enzymes for applied biocatalysis, *Trends Biotechnol.*, 21, 474–478, 2003.
61. Alexeeva, M., Carr, R., and Turner, N.J., Directed evolution of enzymes: new biocatalysts for asymmetric synthesis, *Org. Biomol. Chem.*, 1, 4133–4137, 2003.
62. Carr, R., Alexeeva, M., Enright, A., et al., Directed evolution of an amine oxidase possessing both broad substrate specificity and high enantioselectivity, *Angew. Chem. Intl. Ed. Engl.*, 42, 4807–4810, 2003.
63. May, O., Nguyen, P.T., and Arnold, F.H., Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine, *Nat. Biotechnol.*, 18, 317–320, 2000.

64. Van Loo, B., Spelberg, J.H., Kingma, J., et al., Directed evolution of epoxide hydrolase from *A. radiobacter* toward higher enantioselectivity by error-prone PCR and DNA shuffling, *Chem. Biol.*, 11, 981–990, 2004.
65. Reetz, M.T., Bocola, M., Carballeira, J.D., et al., Expanding the range of substrate acceptance of enzymes: combinatorial active-site saturation test, *Angew. Chem. Int. Edn.*, 44, 4192–4196, 2005.
66. Mink, D., Industrial enzymatic C–C bond formation, *Biotrans*, Delft, July 3–8, 2005.
67. Desantis, G., Wong, K., Farwell, B., et al., Creation of a productive, highly enantioselective nitrilase through gene site saturation mutagenesis (GSSM), *J. Am. Chem. Soc.*, 125, 11476–11477, 2003.
68. Davis, C., Grate, J., Gray, D., et al., Enzymatic process for the production of 4-substituted 3-hydroxybutyric acid derivatives, Codexis, Inc., WO04015132, 2005.
69. Davis, C., Grate, J., Gray, D., et al., Enzymatic process for the production of 4-substituted 3-hydroxybutyric acid derivatives and vicinal cyano, hydroxyl substituted carboxylic acid esters, Codexis, Inc., WO05018579, 2005.
70. Grate, J., A Green and Economic Biocatalytic Process for the Key Chiral Intermediate for Atorvastatin. Using Three Biocatalysts Evolved to Enable the Process, ACS Green Chemistry Conference, Washington, June 24, 2005.

31 Biocatalytic Routes to Nonracemic Chiral Amines

Nicholas J. Turner and Reuben Carr

CONTENTS

31.1	Introduction	743
31.2	Kinetic Resolution	743
31.3	Dynamic Kinetic Resolution	747
31.4	Asymmetric Processes	748
31.5	Deracemization	751
31.6	Conclusions	753
	References	754

31.1 INTRODUCTION

Nonracemic chiral amines are an important, but not easily prepared, class of organic molecules. In addition to their value as building blocks for the preparation of pharmaceutical and agrochemical end products (Figure 31.1) [1], they are increasingly used as ligands for asymmetric catalysis [2] and resolving agents for crystallization. As with the manufacture on scale of many fine chemicals, the current trend is to develop catalytic processes for their production, thereby eliminating the need for the stoichiometric use of reagents that is both expensive and environmentally unattractive. Both chemo- and biocatalytic approaches to the preparation of nonracemic chiral amines are currently being explored, with the former relying to a large extent upon technologies based on the asymmetric hydrogenation of imines [3,4]. In terms of biocatalysis there are essentially three distinct routes by which optically active amines can be prepared: (i) kinetic resolutions of the corresponding racemates, including dynamic kinetic resolution processes; (ii) asymmetric transamination of ketone precursors; and (iii) deracemization reactions. The aim of this chapter is to highlight the various approaches that are being investigated and provide some examples of different routes by which optically active amines can be prepared. The application of some of these routes for the preparation of chiral amines at industrial scale is also discussed with relevant examples [5].

31.2 KINETIC RESOLUTION

While the use of hydrolytic enzymes (e.g., lipases, esterases, proteases) for the kinetic resolution of racemic chiral alcohols and carboxylic acids is well established, the corresponding transformation on racemic amines is less well studied, although it is now starting to receive considerably more attention [6,7]. By far the most commonly used hydrolase for this purpose is *Candida antarctica* lipase B (CAL-B) and, in addition, most of the reported examples involve acylation of racemic chiral amines under low water conditions. One issue that needs

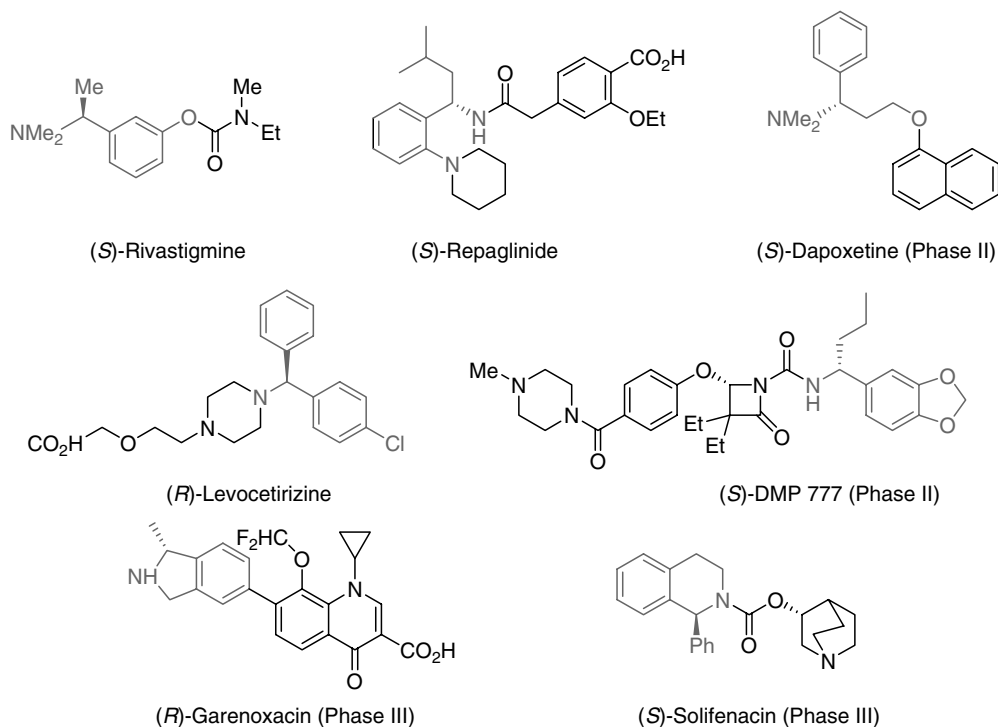


FIGURE 31.1 Pharmaceutical drugs containing chiral amine building blocks (grey).

to be addressed is that amines are more nucleophilic than alcohols and hence are more prone to undergo background reaction with the acyl donor, leading to lower overall enantiomeric excesses (ees) for the product. The first example of this type of reaction was described by Klivanov et al. in 1989, in which they screened a number of hydrolytic enzymes for their ability to catalyze the acylation of racemic α -methyl benzylamine and other chiral amines using trifluoroethyl butyrate as the acylating agent [8]. Interestingly they found that there was a strong dependence of the enantioselectivity on the solvent, with 3-methyl-3-pentanol giving the highest selectivity. Using subtilisin as the catalyst they reported ees up to 99%.

Thereafter, several other groups began to examine the scope of this reaction and also investigate various enzyme/solvent/acyl donor combinations in order to optimize conversions and enantioselectivities. Schneider et al. [9] employed CAL-B for the resolution of various aryl alkynyl amine derivatives **1**, obtaining very high selectivities ($E > 100$) in a number of cases to yield the corresponding (*R*)-amine **1** and (*S*)-amide **2**. The chiral (*R*)-amine products were subsequently used as building blocks for the preparation of a range of antifungal aromatase inhibitors **3** (Figure 31.2).

Likewise, in another pharmaceutical application, researchers at Pfizer Inc. in San Diego demonstrated that CAL-B could be used for the resolution of a simple chiral building block **4**, which was used as an intermediate for the synthesis of cyclin-dependent kinase (CDK2) inhibitors (Figure 31.3) [10].

1,1'-Binaphthylamine derivatives are useful chiral ligands for various asymmetric reactions: 2,2'-diamino-1,1'-binaphthyl has been used as the starting material for the synthesis of BINAP, which is a chiral ligand used in the asymmetric hydrogenation of ketones [11,12]. The lipase-catalyzed acylation of amine **5** was recently reported using LIP-300 (*Pseudomonas aeruginosa* lipase immobilized on Hyflo Super-Cel) and LPL-311 (*P. aeruginosa* lipase on

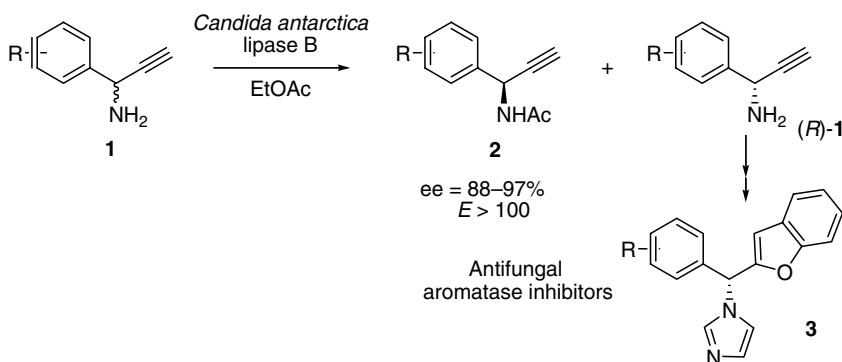


FIGURE 31.2 Resolution of amine building blocks for aromatase inhibitors.

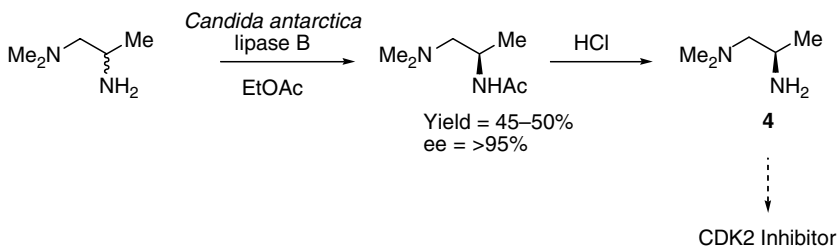
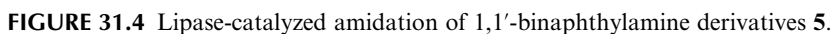


FIGURE 31.3 Chiral amine building blocks for CDK2 inhibitors obtained by CAL-B catalyzed kinetic resolution.

Toyonite 200-M) for the kinetic resolution of 1,1'-binaphthylamine derivatives (Figure 31.4). The amidation reaction was sensitive to the length of the alkyl chain between the binaphthyl ring and amino group, and improved enantioselectivities were observed for increasing alkyl chain length of the (*R*)-substituent on the acyl donor.

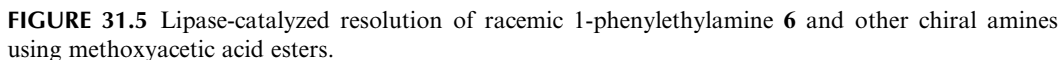
In 1993, BASF reported the results of an extensive screening of lipase-catalyzed acylations of amines, particularly with respect to the nature of the acyl donor. It was found that racemic α -methyl benzylamine **6** could be acylated with *Burkholderia plantarii* lipase with esters of methoxyacetic acid **7**, giving optically pure (*S*)-amine **6** and (*R*)-amide **8** [13] (Figure 31.5). After separation by distillation, the (*R*)-amine was released by basic hydrolysis of the amide without racemization, in quantitative yield. The attractive feature of this process was the broad substrate tolerance of the catalyst and a wide variety of amines have been resolved, in some cases on a multiton scale [14]; recycling of the undesired enantiomer by racemization and recovery of the acylating agent help to lower the cost of the industrial process.

Optically active amines can also be accessed by exploiting alternative hydrolytic enzymes such as amidases and acylases. Compared to lipases, however, these enzymes are somewhat restricted in terms of commercial availability but many are found in nature and can be isolated as microbial cultures. Growing microorganisms in simple growth media containing an amide as the sole carbon and/or nitrogen source for growth selects active microorganisms over the remainder of the population for amidase. Following successive subculturing, the microbial population becomes enriched in microorganisms possessing the desired amidase activity. Using this approach with *N*-acetyl-2-butylamine as the amide substrate, researchers at Avecia Ltd. isolated ~60 microorganisms and found that *Arthrobacter* sp. predominated [15].



Sheldon et al. have also exploited acylases in the kinetic resolution of racemic amines, in this case through an acylation approach. Thus, they demonstrated the use of aminoacylase I from *Aspergillus melleus* together with methyl methoxyacetate as the acyl donor for the resolution of 1-amino indane **12** as shown in [Figure 31.7](#) [17]. The reaction was found to proceed with modest selectivity ($E = 9.3$), resulting in an ee of amine (*S*)-**12** of 72% at 53% conversion (Figure 31.7).

Despite the general approaches described for the resolution of primary amines, there are a few reported methods for the lipase-mediated enantioselective acylation of secondary amines. Moreover, the resolutions that are reported tend to suffer from poor yields and/or poor enantioselectivities. The cyclic secondary amine 1-methyltetrahydroisoquinoline **13** (MTQ) is a building block for YH1885 **14**, a potential treatment for gastroesophageal reflux disease



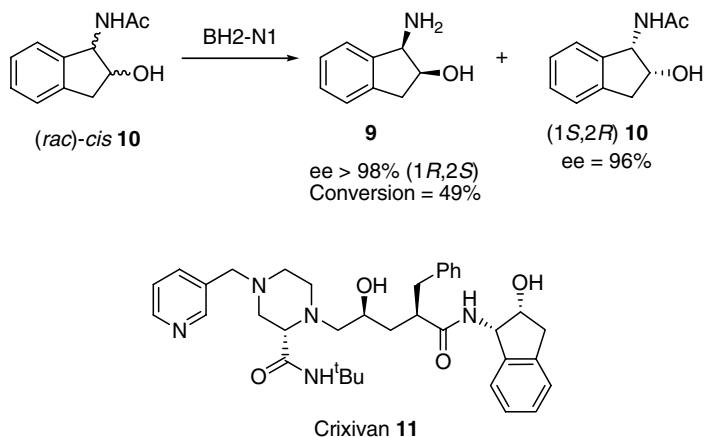


FIGURE 31.6 Kinetic resolution of *N*-acetyl-1-aminoindanol by BH2-N1 amidase. The (1*S*,2*R*)-enantiomer of 1-aminoindanol (shown in grey) is a key intermediate in Indinavir-**11** (Crixivan, Merck).

(GERD) and duodenal ulcers [18]. Researchers at GlaxoSmithKline required access to both enantiomers for the development of the drug. In this example, the kinetic resolution was achieved using substituted phenyl allyl carbonates **15** as the acyl donors. Enantioselective acylation of (*rac*)-MTQ **13** with *C. rugosa* lipase yielded (*S*)-**13** and the corresponding allylcarbamate derivative **16** (Figure 31.8) [19].

31.3 DYNAMIC KINETIC RESOLUTION

Enzyme-catalyzed dynamic kinetic resolution (DKR) reactions, in which the unreacted enantiomer is racemized *in situ*, have been successfully applied in the arena of chiral α -amino acids, secondary alcohols, and carboxylic acids [20]. The DKR of chiral amines has proved more problematic and challenging, principally because chiral amines require much higher temperature for racemization, leading to conditions that are generally incompatible with most hydrolases. The catalytic cycle for a DKR is shown in Figure 31.9, in which it is essential to identify methods for selective racemization of the substrate but not of the product.

The first reported example of DKR of an amine derives from Reetz and Schimossek in which they employed palladium as a catalyst for the racemization of α -methyl benzylamine in the presence of CAL-B [21]. The reaction was carried out in a solution of pyridine, with ethyl acetate as the acyl donor, at a temperature of 50 to 55°C. In order to achieve total conversion

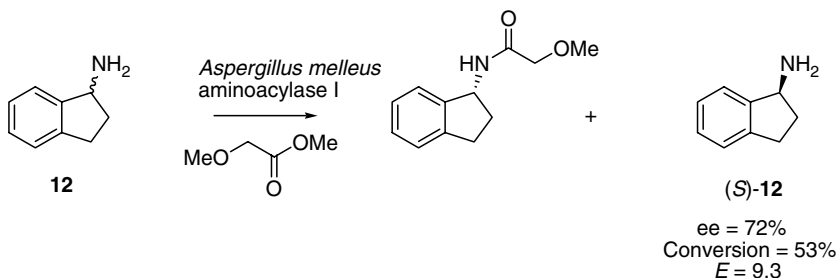


FIGURE 31.7 Resolution of rac-1-aminoindane **12** to (*S*)-1-aminoindane.

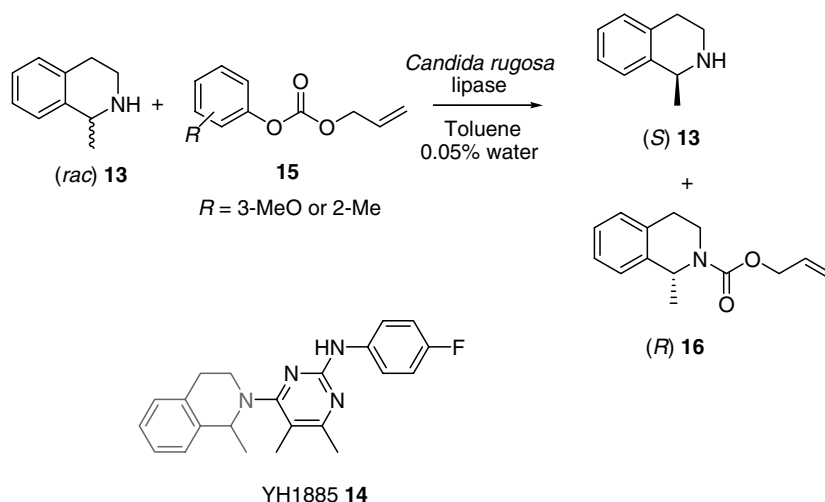


FIGURE 31.8 Lipase-catalyzed kinetic resolution of MTQ **13** with a phenylallyl carbonate acyl donor **15**. MTQ is a building block (shown in grey) in the drug candidate YH1885 **14**.

to the *N*-acylated product it was necessary to leave the reaction for 5 d giving the (*R*)-amide in 64% yield and 99% ee. Kim et al. [22] extended this approach by using the *in situ* reduction of the corresponding oxime **17** to the racemic amine, thereby avoiding the presence of high concentrations of the amine at the beginning of the reaction (Figure 31.10).

Backvall has recently taken this area off into a new direction by examining the use of ruthenium-based catalysts for the racemization of amines (Figure 31.11). Such catalysts have previously been shown to be effective for the DKR of secondary alcohols in combination with CAL-B and subtilisin as biocatalysts. However, as discussed above, the rates of racemization with chiral amines are substantially reduced; hence, the reactions require higher temperatures.

In fact, the initial work involving Shvo's catalyst **18** required a reaction temperature of 110°C, which precluded the *in situ* use of the CAL-B [23]. However, very recently Backvall has shown that catalyst **19**, in which the phenyl substituents on the cyclopentadiene ring are replaced by *para*-methoxyphenyl groups, is able to catalyze efficient amine racemization at a lower temperature (90°C). At this lower temperature, it is now possible to combine the biocatalyst (CAL-B) with the transition metal catalyst to effect a true DKR of amines [24].

31.4 ASYMMETRIC PROCESSES

Transaminases have also been used in the production of optically active amines. In this process, a carbonyl compound such as a ketone **20** or an α -keto acid is converted directly into an amine (Figure 31.12). The enantioselective transfer is dependent on the transaminase

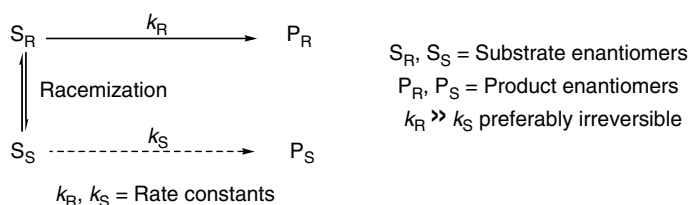


FIGURE 31.9 Reaction scheme for a DKR.

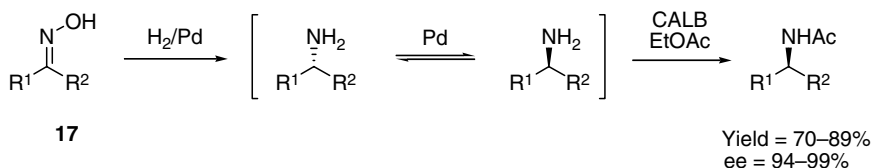


FIGURE 31.10 DKR of racemic chiral amines using palladium.

cofactor pyridoxal phosphate **23**. Celgene has developed both (*S*)- and (*R*)-selective transaminases, enabling both enantiomers to be accessed [25]. The reaction can be carried out either in the synthesis mode (route A) or as a kinetic resolution (route B) (Figure 31.12). In the synthetic procedure isopropyl amine **21** provides an amino group, which is transferred to a prochiral ketone enantioselectively to give the optically active amine product plus acetone **22**. The kinetic resolution is essentially the synthesis in reverse, where the racemic amine substrate (e.g., **6**) is enantioselectively converted to the ketone. The amino group is transferred to a low-molecular weight aldehyde such as propionaldehyde **24**. If α -keto acids are used in place of ketones, amino acids are obtained.

Stirling et al., [26] found that the wild-type enzymes possessed low activity and enantioselectivity but it was able to improve these properties by successive rounds of random mutagenesis and screening (directed evolution) such that the ee could be improved to >99%. It also employed directed evolution to improve the tolerance of the transaminase enzymes to high amine concentration, leading to improvement in the process development for large-scale production of amines. For example, it reported pilot-scale production of amines up to multi-100 kg scale and also fermentation of *Escherichia coli* cells expressing the enzymes from 20 L to several m³ capacity. An example of the range of chiral amines accessible with this technology is shown in Figure 31.13.

Kim et al. [27,28] have recently reported, the use of an ω -transaminase from *Vibrio fluvialis* as shown in Figure 31.14. In order to shift the equilibrium toward formation of the product

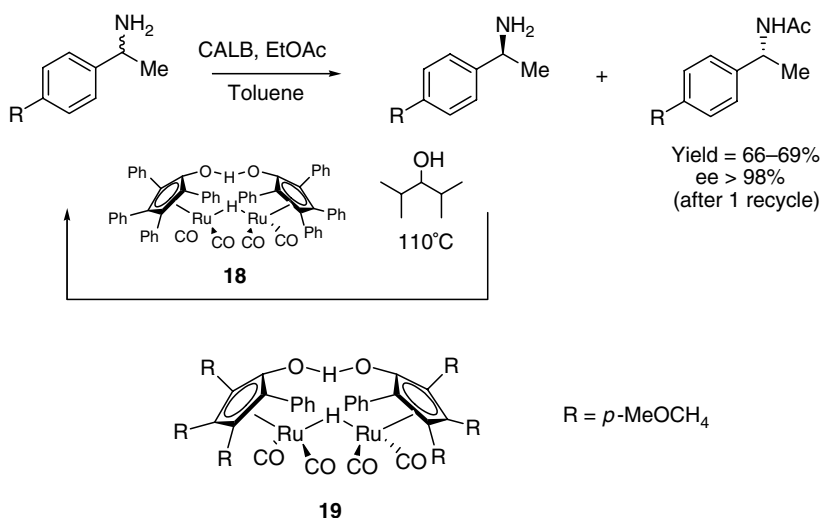
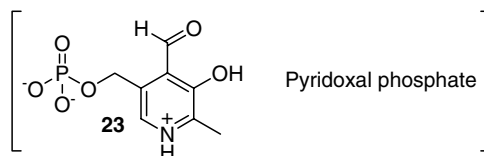
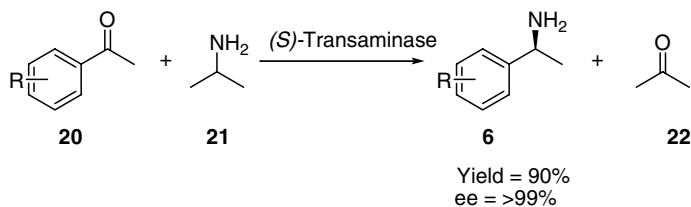


FIGURE 31.11 DKR of α -methyl benzylamine using Shvo's catalyst **18** and analog **19**.

Route A



Route B

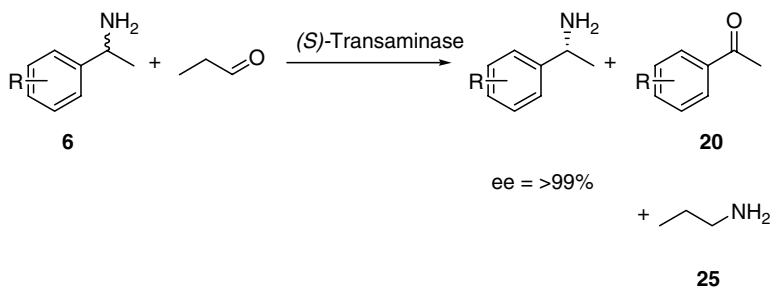


FIGURE 31.12 Route A is the synthesis procedure for the transamination of a prochiral ketone **20** using an (*S*)-transaminase and isopropylamine. Route B is the kinetic resolution procedure of a (rac)-amine **6** to the (*R*)-amine **6** (Celgene).

amine they employed excess L-alanine as the amino donor. However, they also found that the reaction was inhibited by the formation of pyruvate. This problem could be overcome by the addition of lactate dehydrogenase, which converted the pyruvate to lactate. By using whole cells and a onefold excess of alanine they were able to obtain the product amine **6** in ~90% yield in less than 24 h.

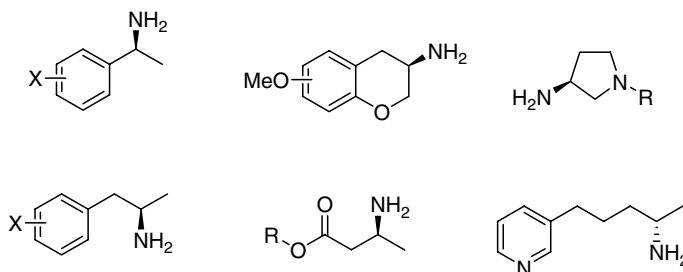


FIGURE 31.13 Chiral amines accessible with transaminase technology.

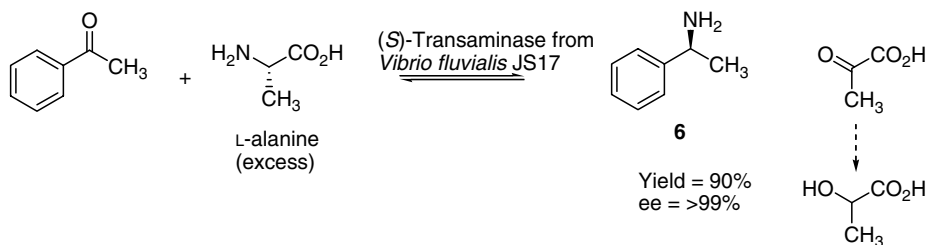


FIGURE 31.14 Transaminase from *Vibrio fluvialis*.

31.5 DERACEMIZATION

Deracemization is a process during which a racemate is converted into a nonracemic product in 100% theoretical yield without intermediate separation of materials. According to this definition, DKR, dynamic thermodynamic resolution, stereoinversion, and enantioconvergent transformations of a racemate are all classified as deracemization processes [29]. In principle, a cyclic oxidation and reduction sequence as shown in Figure 31.15 can lead to the deracemization of chiral alcohols and amines.

This process relies upon the enantioselective oxidation of the substrate ($k_R \gg k_S$) such that one enantiomer of the starting racemate is oxidized to the achiral intermediate, either a ketone or imine. The oxidized product is then converted back to the alcohol or amine by a chemical reduction in a nonselective manner.

The first example of such a process originates from Hafner and Wellner, who reported the generation of L-alanine and L-leucine from the corresponding D-enantiomers by the use of porcine kidney D-amino acid oxidase and sodium borohydride [30]. Subsequently, Soda et al. [31,32] extended this method for the deracemization of DL-proline and DL-pipecolic acid also using D-amino acid oxidase and sodium borohydride (Figure 31.16).

It is interesting to note that even after just four cycles of oxidation and reduction, the ee of L-amino acid is >93% from a starting racemate assuming a completely enantioselective oxidation and nonselective chemical reduction. By seven cycles the ee has risen to >99% (Figure 31.17).

Beard and Turner [33] have recently considerably expanded the scope and application of the deracemization of amino acids including the discovery of novel reducing agents such as sodium cyanoborohydride, amine-borane complexes, and catalytic transfer hydrogenation [34] that can be used in place of sodium borohydride. The use of catalytic transfer hydrogenation has also shown to be effective as the reducing agent in the deracemization of amino

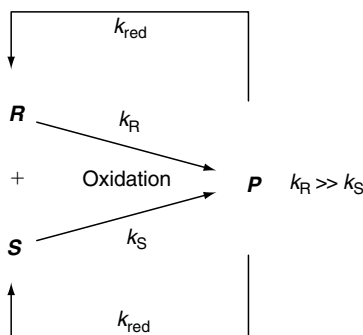


FIGURE 31.15 Deracemization by a cyclic oxidation and reduction sequence.

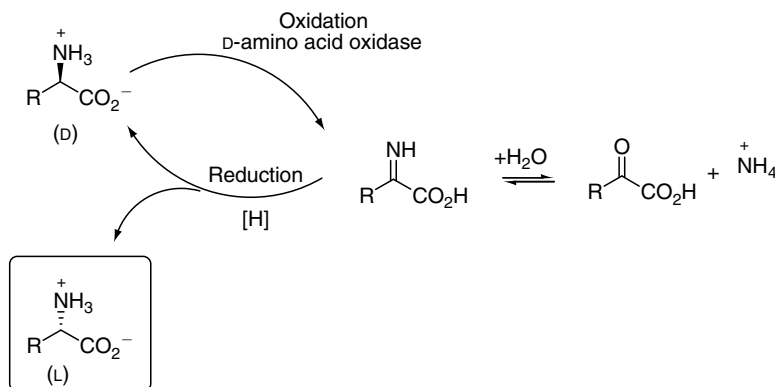


FIGURE 31.16 Deracemization of DL-amino acids to D-amino acids using D-amino acid oxidase combined with a chemical reducing agent.

acids with L-amino acid oxidase giving D-amino acids [34]. Extension of the deracemization approach of α -amino acids has been used in the stereoinversion of β - and γ -substituted α -amino acids using the chemo-enzymatic oxidation and reduction procedure [35,36].

In a further recent development, the concept of deracemization by an oxidation and reduction sequence has been applied to chiral amines. In a key proof-of-principle experiment, Turner et al. [37] were able to demonstrate successfully the deracemization of α -methyl benzylamine **6** in 77% yield and 93% ee. The amine oxidase required for this process was obtained from *A. niger*. However, the wild-type enzyme had very narrow substrate specificity, typically reacting with simple achiral amines such as amyl amine, butyl amine, and benzyl amine. In order to improve the activity of the wild-type enzyme toward chiral amines such as α -methyl benzylamine **6**, the amine oxidase was subjected to an initial round of directed evolution using a high-throughput colorimetric screen on solid phase. A single mutant (Asn336Ser) was identified with considerably enhanced (~50-fold) activity toward α -methyl benzylamine. Interestingly this mutant was also found to have a much broader substrate specificity than the parent and catalyzed the oxidation of a wide range of structurally different chiral amines (Figure 31.18) [38]. In all cases examined, the enzyme was found to have high (*S*)-selectivity. Subsequently,

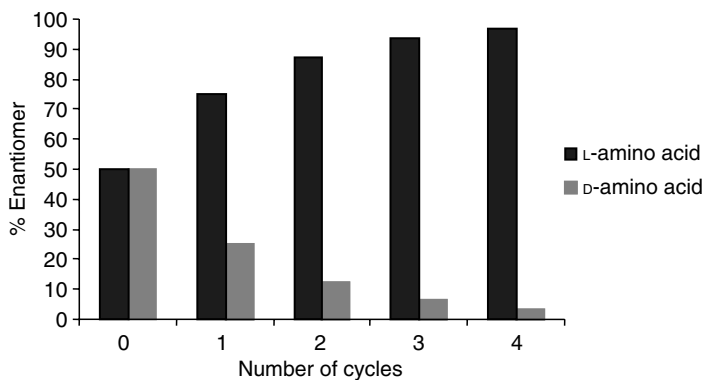


FIGURE 31.17 Enantiomeric excess as a function of number of catalytic cycles during the deracemization process.

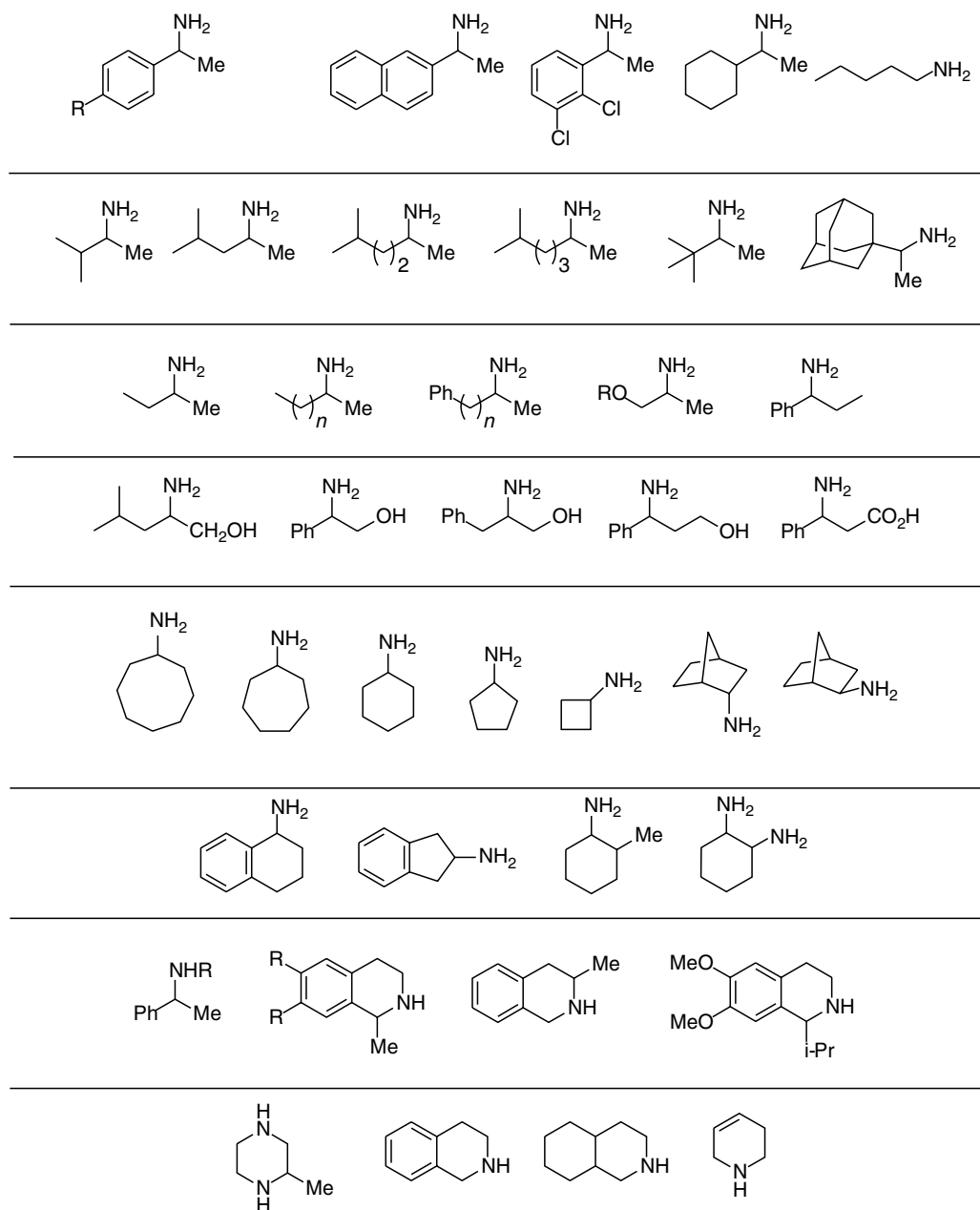


FIGURE 31.18 Chiral amines that are substrates for double mutants of amine oxidase.

a second mutation was also reported (Ile246Met) which further enhanced the activity of the amine oxidase toward chiral cyclic secondary amines as exemplified by the preparative deracemization of MTQ (Figure 31.19) [39].

31.6 CONCLUSIONS

Chiral, nonracemic amines are increasingly in demand, particularly as building blocks for the preparation of pharmaceutical and agrochemical products. Currently, there are relatively few

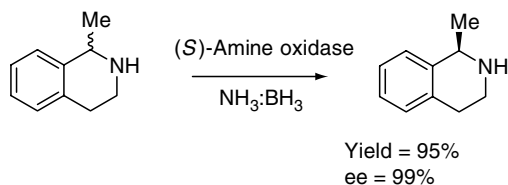


FIGURE 31.19 Deracemization of MTQ.

options for their preparation using biocatalytic approaches, particularly in comparison with chiral secondary alcohols in which the use of hydrolytic enzymes or dehydrogenases offer relatively mature technologies. However, significant advances are being made in a number of areas as outlined above and it is likely that each of the approaches described will become increasingly competitive as a means of preparing chiral amines on scale.

REFERENCES

- Breuer, M., Ditrich, K., Habicher, T., Hauer, B., Kessler, M., Stuermer, R., and Zelinski, T., *Angew. Chem. Int. Ed.*, 43, 788, 2004.
- Jacobsen, E.N., Pfaltz, A., and Yamamoto, H., in *Comprehensive Asymmetric Catalysis*, Eds., Springer, Berlin, 1999.
- Boezio, A.A., Pytkowicz, J., Côté, A., and Charette, A.B., *J. Am. Chem. Soc.*, 125, 14260, 2003.
- Williams, G.D., Pike, R.A., Wade, C.E., and Wills, M., *Org. Lett.*, 5, 4227, 2003.
- Turner, N.J., Fotheringham, I., and Speight, R., *Innov. Pharm. Technol.*, 4, 114, 2004.
- For a recent review, see: van Rantwijk, F., and Sheldon, R.A., *Tetrahedron*, 60, 501, 2004.
- García-Urdiales, E., Alfonso, I., and Gotor, V., *Chem Rev.*, 105, 313, 2005.
- Kitaguchi, H., Fitzpatrick, P.A., Huber, J.E., and Klibanov, A.M., *J. Am. Chem. Soc.*, 111, 3094, 1989.
- Messina, F., Botta, M., Corelli, F., Schneider, M.P., and Fazio, F.J., *J. Org. Chem.*, 64, 3767, 1999.
- Tao, J., Chiral USA, Scientific Update, October 2003, Chicago.
- Noyori, R., *Adv. Synth. Catal.*, 345, 15, 2003.
- Aoyagi, N. and Izumi, T., *Tetrahedron Lett.*, 43, 5529, 2002.
- Hieber, G. and Ditrich, K., *Chimica Oggi*, 16, 2001.
- Balkenhohl, F., Ditrich, K., Hauer, B., and Ladner, W., *J. Prakt. Chem.*, 339, 381, 1997.
- Reeve, C.D., Holt, R.A., Rigby, S.R., and Hazel, K., *Chimica Oggi*, 19, 31, 2001.
- Senanayake, C.H., *Aldrichimica Acta*, 31, 3, 1998.
- Youshko, M.I., van Rantwijk, F., and Sheldon, R.A., *Tetrahedron Asymmetry*, 12, 3267, 2001.
- Lee, J.W., Chae, J.S., Kim, C.S., Kim, J.K., Lim, D.S., Shon, M.K., Choi, Y.S. and Lee, S.H., Patent WO9605177, 1996.
- Breen, G.F., *Tetrahedron Asymmetry*, 15, 1427, 2004.
- Turner, N.J., *Curr. Opin. Chem. Biol.*, 8, 114, 2004.
- Reetz, M.T. and Schimossek, K., *Chimia*, 50, 668, 1996.
- Choi, Y.K., Kim, M.-J., Ahn, Y., and Kim, M.J., *Org. Lett.*, 3, 4099, 2001.
- Pamies, O., Ell, A.H., Samec, J.S.M., Hermanns, N., and Bäckvall, J.E., *Tetrahedron Lett.*, 43, 4699, 2002.
- Paetzold, J. and Bäckvall, J.E., *J. Am. Chem. Soc.*, 127, 17620, 2005.
- Stewart, J.D., *Curr. Opin. Chem. Biol.*, 5, 120, 2001.
- Stirling, D.I., Zeitlin, A., and Matcham, G.W., 1990, US Patent 4,950,606; see also www.cambrex.com.
- Hwang, B.-Y., Cho, B.-K., Yun, H., Koteswar, K., and Kim, B.-G., *J. Mol. Catal. B Enzym.*, 37, 47, 2005.
- Shin, J.-S. and Kim, B.-G., *Biotechnol. Bioeng.*, 65, 206, 1999.

29. Faber, K., *Chem. Eur. J.*, 7, 5005, 2001.
30. Hafner, E.W. and Wellner, D., *Proc. Nat. Acad. Sci.*, 68, 987, 1971.
31. Huh, J.W., Yokoigawa, K., Esaki, N., and Soda, K., *J. Ferment. Bioeng.*, 74, 189, 1992.
32. Huh, J.W., Yokoigawa, K., Esaki, N., and Soda, K., *Biosci. Biotech. Biochem.*, 56, 2081, 1992.
33. Beard, T. and Turner, N.J., *Chem. Commun.*, 246, 2002.
34. Alexandre, F.-R., Pantaleone, D.P., Taylor, P.P., Fotheringham, I.G., Ager, D.J., and Turner, N.J., *Tetrahedron Lett.*, 43, 707, 2002.
35. Enright, A., Alexandre, F.-R., Roff, G., Fotheringham, I.G., Dawson, M.J., and Turner, N.J., *Chem. Commun.*, 2636, 2003.
36. Roff, G.J., Lloyd, R.C., and Turner, N.J., *J. Am. Chem. Soc.*, 126, 4098, 2004.
37. Alexeeva, M., Enright, A., Dawson, M.J., Mahmoudian, M., and Turner, N.J., *Angew. Chem. Int. Ed.*, 41, 3177, 2002.
38. Carr, R., Alexeeva, M., Enright, A., Eve, T.S.C., Dawson, M.J., and Turner, N.J., *Angew. Chem. Int. Ed.*, 42, 4807, 2003.
39. Carr, R., Alexeeva, M., Dawson, M.J., Gotor-Fernández, V., Humphrey, C.E., and Turner, N.J., *Chem. Bio. Chem.*, 6, 637, 2005.

32 Enantioselective Biocatalytic Reduction of Ketones for the Synthesis of Optically Active Alcohols

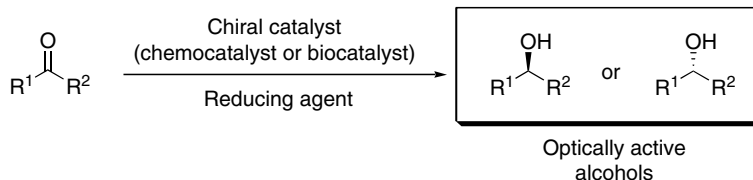
Stefan Buchholz and Harald Gröger

CONTENTS

32.1	Introduction	757
32.2	The Concepts of Biocatalytic Reductions of Ketones.....	758
32.3	Processes Based on Substrate-Coupled Cofactor Regeneration.....	760
32.3.1	The Concept.....	760
32.3.2	Processes with Isolated Enzymes.....	761
32.3.3	Whole-Cell Reductions	763
32.4	Processes Based on Enzyme-Coupled Cofactor Regeneration with a Formate Dehydrogenase.....	768
32.4.1	The Concept.....	768
32.4.2	Processes with Isolated Enzymes.....	770
32.4.3	Whole-Cell Reductions	775
32.5	Processes Based on Enzyme-Coupled Cofactor Regeneration using a Glucose Dehydrogenase.....	777
32.5.1	The Concept.....	777
32.5.2	Processes with Isolated Enzymes.....	778
32.5.3	Whole-Cell Reductions	780
32.6	Processes Based on Cofactor Regeneration with Glucose-6-Phosphate Dehydrogenase.....	784
32.6.1	The Concept.....	784
32.6.2	Isolated Enzymes.....	785
32.6.3	Whole-Cell Reductions	786
32.7	Summary and Outlook.....	786
	References and Notes.....	787

32.1 INTRODUCTION

The enantioselective reduction of ketones—according to [Scheme 32.1](#)—represents both a straightforward and an atom-economical approach toward optically active alcohols, which are important building blocks, for example, for the production of pharmaceuticals [1]. Often so-called blockbusters, drugs that are marketed with sales in the billion US\$ range, are based on the



SCHEME 32.1

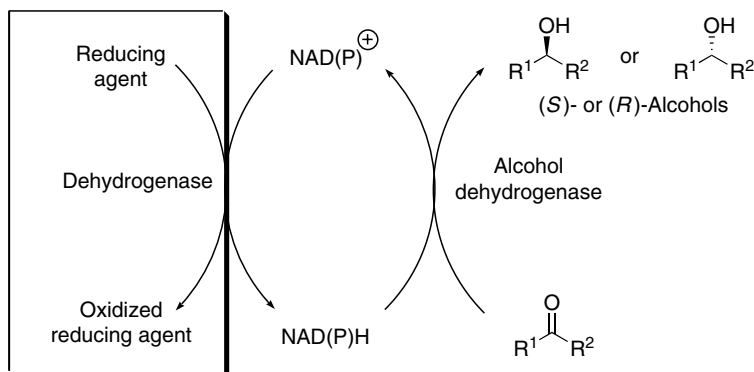
use of a chiral alcohol moiety. Thus, it is not surprising that numerous efficient asymmetric catalytic routes based on different types of concepts—from the fields of kinetic resolution [2] and asymmetric synthesis [3–5]—have been developed up to date. Without any doubt, outstanding technologies in the latter field are the metal-catalyzed asymmetric hydrogenation of ketones [6] and the borane reduction [7]. Both technologies, which can be regarded as landmarks in industrial asymmetric catalysis in general, are applied widely on technical scale, and certainly represent the benchmark for any other type of alternative catalytic reduction methodology.

However, biocatalysis [8] has turned out more and more to be an alternative, highly competitive technology for asymmetric ketone reductions. The recent increase in the number of industrial applications of the biocatalytic asymmetric ketone reductions underlines the tremendous potential of this type of “white biotechnology” for large-scale manufacture of enantiomerically pure alcohols. Notably, the “biocatalytic reduction of ketones” is not just one type of reaction but rather consists of a range of different, often complementary, concepts.

The goal of this review is to cover the state of the art in the field of enantioselective biocatalytic reductions of ketones for the synthesis of optically active alcohols (for previous reviews, see references [9–14]). The major focus is on those methods that have already demonstrated their feasibility on preparative and/or technical scale. In this connection, the industrial impact of the different enzymatic approaches is discussed, and selected applications for large-scale manufacture of chiral alcohols are presented. The review is subdivided according to the different concepts of cofactor regeneration, which is a key issue for achieving economically attractive processes. In the scope of this review, typical yeast whole-cell processes (which have been reviewed extensively elsewhere; see, for example, [15–19]), “fermentation-type processes” using resting cells under metabolism of glucose for cofactor regeneration [20], and diastereoselective reductions, for example, of water-soluble sugar-type carbonyl compounds [21] are not included. With respect to technical applications it should be noted that, for example, diastereoselective biocatalytic reduction is already applied on industrial scale at Avecia Ltd. for the synthesis of (4*S*,6*S*)-5,6-dihydro-4-hydroxy-6-methyl-4*H*-thieno[2,3*b*]thiopyran-7,7-dioxide, starting from the corresponding enantiomerically pure (6*S*)-ketone [22,23]. Therein this pharmaceutically important alcohol is manufactured by the wild-type strain *Neurospora crassa*, obtaining high yield (>85%) and enantioselectivity (>98% ee) [24].

32.2 THE CONCEPTS OF BIOCATALYTIC REDUCTIONS OF KETONES

The basic principle of the enantioselective biocatalytic reduction of ketones (Scheme 32.2) is the use of an alcohol dehydrogenase (ADH) as a catalyst, and a cofactor as a reducing agent. An ADH is an enzyme capable of reducing carbonyl moieties under formation of (chiral) alcohols and requires a specific “cofactor” as reducing agent. The most preferred cofactors are either NADH or NADPH, respectively. As the cofactors are very expensive reducing agents, and too costly to be applied in stoichiometric amount, a common key feature of all preparative (and technical) biocatalytic reductions is the use of cofactors in low, even catalytic, amounts, and their



SCHEME 32.2

recycling *in situ* by coupling the ketone reduction process with a second process, in which the cofactor is regenerated (Scheme 32.2). Accordingly, the efficiency of regeneration of the cofactor contributes directly to the process economy of the whole reduction process and its synthetic applicability.

Notably, there are several options for an *in situ* cofactor recycling. In particular, substrate-coupled cofactor regeneration (Section 32.3), and enzyme-coupled cofactor regeneration (Section 32.4 through Section 32.6) have been developed. With respect to the latter, established cofactor regenerations based on formate dehydrogenase (FDH), glucose dehydrogenase (GDH), and glucose-6-phosphate dehydrogenase (G6PDH) are covered by this review. It should be added at this stage that alternative enzymatic cofactor regeneration concepts based on, for example, hydrogenase [25], phosphate dehydrogenase [26], and malic enzyme [27], have also been developed, which, albeit promising, are not covered by this review. This is in part because so far data from synthetic application of these methods are still rare, and also because there are no technical applications yet. In addition, electrochemical cofactor regeneration is known [28], but less commonly applied in asymmetric synthesis of alcohols compared to the above-mentioned methodologies. A common feature of all these types of cofactor regenerations is the use of a cheap and easily available source for the reduction of the oxidized form of the cofactor, namely NAD^+ or NADP^+ . In case of the substrate- and enzyme-coupled cofactor regenerations, this source is a cheap and easily available reducing agent such as 2-propanol in the first case, and formate or glucose in the latter one.

The second key success factor is related to the properties of the ADHs. Although numerous ADHs are known, a multitude of biocatalytic reductions are based on the use of a relatively limited range of enzymes. Some selected examples of ADHs, which are widely applied in enzymatic reduction of ketones, are shown in Table 32.1. The synthetic applications of these enzymes are discussed in more detail in subsequent sections. It is noteworthy that the majority of these enzymes are already available in recombinant form by means of expression in *Escherichia coli*. These enzymes have already been often synthetically applied as they fulfil the prerequisites for synthetic applicability, for example, with respect to availability, high activity, and selectivity as well as a broad substrate tolerance. The variety of ADHs available is tremendous. This has been impressively demonstrated by, for example, Homann et al. in a broad ADH screening for about 30 ketones with a microbial library of about 300 microorganisms, identifying 60 cultures that contain highly enantioselective ADHs [29]. It should be added that several ADHs have already been characterized with respect to their stability in organic solvents. For example, a detailed study has been done by Müller et al. investigating the compatibility of solvents with ADHs from horse liver, *Thermoanaerobium Brockii*, and *Lactobacillus brevis* [30].

TABLE 32.1**Selected Alcohol Dehydrogenases (ADHs) That Have Been Used in the Asymmetric Reduction of Ketones**

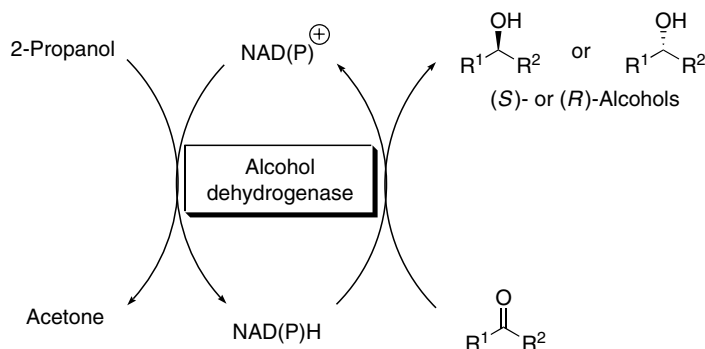
Organism	Cofactor	Enantiospecificity	Source
<i>Acinetobacter calcoaceticus</i>	NADH	(<i>R</i>)	Wild-type organism
Baker's yeast (19 different ADHs)	NADPH	Both (<i>S</i>) and (<i>R</i>)	Recombinant in <i>Escherichia coli</i>
<i>Candida boidinii</i>	NADH	(<i>S</i>)	Wild-type organism
<i>C. magnoliae</i>	NADPH	(<i>R</i>)	Recombinant in <i>E. coli</i>
<i>C. parapsilosis</i>	NADH	(<i>S</i>)	Recombinant in <i>E. coli</i>
<i>Corynebacterium</i> strain ST-10	NADH	(<i>S</i>)	Recombinant in <i>E. coli</i>
<i>Geotrichum candidum</i>	NADH	(<i>S</i>)	Wild-type organism
<i>Gluconobacter oxydans</i>	NADH	(<i>S</i>)	Wild-type organism
<i>Hansenula polymorpha</i>	NADPH	(<i>R</i>)	Recombinant in <i>E. coli</i>
Horse liver	NADH	(<i>S</i>)	Isolated from horse liver
<i>Leifsonia</i> sp.	NADH	(<i>R</i>)	Wild-type organism
<i>Lactobacillus kefir</i>	NADPH	(<i>R</i>)	Recombinant in <i>E. coli</i>
<i>L. kefir</i>	NADPH	(<i>R</i>)	Recombinant in <i>E. coli</i>
<i>L. minor</i>	NADPH	(<i>R</i>)	Recombinant in <i>E. coli</i>
<i>Pichia finlandica</i>	NADH	(<i>S</i>)	Recombinant in <i>E. coli</i>
<i>P. methanolica</i>	NADPH	(<i>S</i>)	Recombinant in <i>E. coli</i>
<i>Pseudomonas</i> sp.	NADH	(<i>R</i>)	Wild-type organism
<i>Pseudomonas fluorescens</i>	NADH	(<i>R</i>)	Recombinant in <i>E. coli</i>
<i>Rhodococcus erythropolis</i>	NADH	(<i>S</i>)	Recombinant in <i>E. coli</i>
<i>R. ruber</i>	NADH	(<i>S</i>)	Recombinant in <i>E. coli</i>
<i>Sporobolomyces salmonicolor</i>	NADPH	(<i>S</i>)	Recombinant in <i>E. coli</i>
<i>Thermoanaerobium brockii</i>	NADPH	Both (<i>S</i>) and (<i>R</i>)	Recombinant in <i>E. coli</i>

The ADHs can be used as isolated enzymes (in purified form or as a crude extract) or as whole cells. With respect to the latter approach, the use of wild-type cells or recombinant whole-cell organisms, called “designer bugs,” is conceivable. Recently, the tailor-made whole-cell catalysts, bearing the ADH and (in case of the enzyme-coupled cofactor regeneration) an additional enzyme, gained more and more interest due to their beneficial properties. Due to overexpression, the desired enzymes are available within the cells in large amount, thus avoiding undesired side reactions by other dehydrogenases and allowing an economically attractive access to them, particularly when using high-cell density fermentation for biocatalyst production.

32.3 PROCESSES BASED ON SUBSTRATE-COUPLED COFACTOR REGENERATION

32.3.1 THE CONCEPT

In the asymmetric reduction of ketones to alcohols according to the concept of substrate-coupled cofactor regeneration, a second alcohol is used as reducing agent for the oxidized cofactor. Typically the same enzyme, namely the same ADH, catalyzes both the formation of the product and the regeneration of the cofactor ([Scheme 32.3](#)). The substrate-coupled cofactor regeneration bears a special potential for the application of NAD(P)H-dependent



SCHEME 32.3

ADHs due to the possibility to use only one enzyme for both required transformations [31]. Another big advantage and the simplicity of this concept is that only one enzyme has to be produced, and often in the applied whole-cell systems not even the addition of an “external” cofactor, which represents an additional cost factor, is necessary. The reaction concept is the biotechnological analog to the classical Meerwein–Ponndorf–Verley reduction that has been known since the 1920s. Simple alcohols like 2-propanol are cheap, environment-friendly, and also claim to be good examples for green chemistry [32]. A principal drawback of this concept, however, is also obvious: as we are dealing with an equilibrium reaction, a huge excess of reducing alcohol has to be added in order to reach high conversion rates. This high cosubstrate concentration might lead to an enzyme inhibition and/or inactivation, or only very low concentrations of the reducing alcohol and the substrate can be applied [33]. If industrially relevant substrate concentrations are being used, the necessary concentration of the reducing alcohol often becomes prohibitive. Although this hampered the application of the substrate-coupled cofactor regeneration for a long time, several successful examples of technical applications based on this concept have been reported.

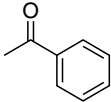
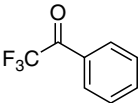
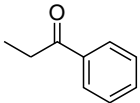
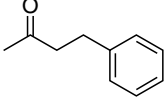

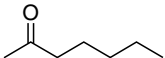
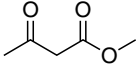
32.3.2 PROCESSES WITH ISOLATED ENZYMES

One of the early works describing the enantioselective reduction of ketones using a substrate-coupled cofactor regeneration was published by Wong et al., who isolated and characterized the ADH from the *Pseudomonas* sp. strain PED [34]. The PED-ADH that utilizes NADH as cofactor has an activity of 36 U/mg with respect to 2-propanol under NAD^+ saturation conditions. As can be seen from Table 32.2 aromatic as well as cyclic and aliphatic ketones are accepted as substrates. Although substrates with a methyl group on one side of the keto-function are typically better substrates for the PED-ADH, other substrates such as ethyl phenylketone are also being accepted.

PED-ADH yields the (*R*)-alcohols in a low to good enantioselectivity (Table 32.3). All reactions have been carried out in a two-phase solvent system with *n*-hexane as organic phase.

Adlercreutz et al. [35] analyzed the influence of temperature and cosubstrate concentration on the enantiomeric excess (ee) of ketone reductions by the NADPH-dependent ADH of *T. Brockii* with 2-propanol as cosubstrate. The reactions were carried out with the immobilized enzyme in hexane with 2.5% (v/v) water, allowing conversions to be carried out at temperatures as low as -20°C . The ee of the alcohol formed from the ketone decreased during the course of reaction (from 53 to 0% in the formation of (*R*)-2-butanol). This is interpreted as being a consequence of the reversibility of all reactions involved. By using a

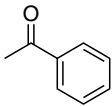
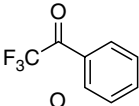
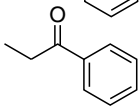
TABLE 32.2
Relative Rate of Reduction of Ketones with PED
Alcohol Dehydrogenase (PED-ADH)

Substrate	Relative Rate of Reaction
	1
	7
	<1
	6
	100
	5
	135

large excess of 2-propanol, this effect was suppressed. While in the reduction of 2-butanone to (*R*)-2-butanol the ee increased with increasing temperature, in the reduction of 2-pentanone to (*S*)-2-pentanol the ee decreased with increasing temperature. This effect, however, could be explained by thermodynamic effects, and one should keep in mind that in the reduction of 2-butanone the (*R*)-alcohol is the predominant product and in the reduction of 2-pentanone (*S*)-alcohol is the main product. An increasing temperature thus favors the formation of the (*R*)-alcohol in both cases.

Specifically for commercial applications besides yield and enantioselectivity, catalyst lifetime and total turnover number are crucial. Margolin et al. [36] reported on the successful stabilization of horse liver ADH–NADH complexes (HLADH–NADH–CLEC) by the formation of glutaraldehyde cross-linked enzyme crystals. While the soluble enzyme rapidly deactivated at 40°C in 25% 2-propanol ($t_{1/2}(40^{\circ}\text{C}) = 4\text{ h}$; $t_{1/2}(25^{\circ}\text{C}) = 24\text{ h}$), the HLADH–NADH–CLEC had a half-life of more than 4 d at 40°C and still showed nearly full activity after 48 h at 25°C. It has been shown that the cofactor is not leaching, which is explained by the enzyme being cross-linked in the cofactor-binding conformation. However, by adding an external cofactor the activity of the crystallized enzyme for the reduction of 6-methyl-5-heptene-2-one could be increased from 64 to 92% of the soluble HLADH activity. A general

TABLE 32.3

Product	Enantiomeric Excess (%)	Yield (%)
	94	34
	98	79
	27	43

limitation of the HLADH, however, is the low specific activity and the nonavailability on a large scale [14].

Gupta et al. [37] reported on the recombinant expression of the NADPH-dependent *L. minor* ADH in *E. coli* and its application in a two-phase system. They were able to quantitatively convert 4-chloro-3-oxobutanoate to the corresponding ethyl (*S*)-chloro-3-hydroxy butanoate with an enantioselectivity of >99.9% ee at a substrate concentration of about 15% (v/v). The enzyme has broad pH stability and a remarkable stability against organic solvents. Some water-miscible solvents even increase stability: e.g., in a 10% solution of glycerol 62% of activity is left after 24 h, while only 3% of activity remains in *tris*-buffer.

The use of a two-phase system composed of a water-immiscible solvent like hexane and a PVA-hydrogel containing *L. kefir* ADH has been shown to be advantageous for many poorly water-soluble substrates by Ansorge-Schumacher et al. [38].

The sequence and cloning of the (*R*)-ADH from *Pseudomonas fluorescens* as well as its use in a cosubstrate-coupled NADH regeneration has been reported by Bornscheuer et al. [39,40]. For acetophenone as a model substrate, 95% conversion and 92% ee have been observed after a reaction time of 21 h using 2-propanol for cofactor regeneration. With methyl-3-oxobutanoate, 83% conversion after 19 h and >99% ee have been obtained.

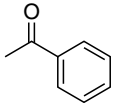
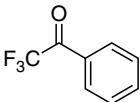
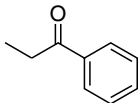
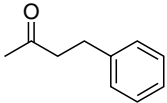

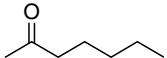
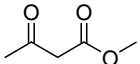
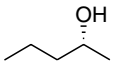
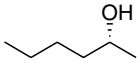
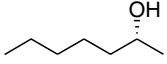
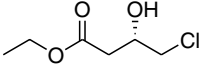
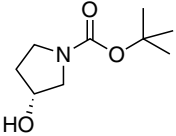
Recently Itoh et al. [41,42] characterized an alcohol dehydrogenase from *Leifsonia* sp. S749 (LSADH) having a broad substrate specificity and showing a high enantioselectivity. All reactions had been carried out with the purified enzyme using 2-propanol for cofactor regeneration (Table 32.4).

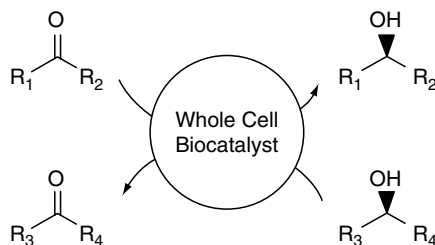
The potential of ionic liquids for the biocatalytic reduction of ketones has been demonstrated by Kragl et al. [43]. Due to the favorable partitioning coefficients the highly enantioselective reduction of 2-octanone to (*R*)-2-octanol, catalyzed by an ADH from *L. brevis*, is faster in a two-phase system containing buffer and the ionic liquid [BMIM][CF₃SO₂N] compared to the reduction in a two-phase system containing buffer and methyl *tert*-butyl ether (MTBE). After a reaction time of 180 min, a conversion of 88% has been observed for the two-phase system with the ionic liquid in contrast to 61% in the presence of MTBE.

32.3.3 WHOLE-CELL REDUCTIONS

In the whole-cell bioreductions of ketones to chiral alcohols (Scheme 32.4), either wild-type cells or recombinant strains may be used. A significant advantage of this reaction concept is

TABLE 32.4

Product	Enantiomeric Excess (%)	Relative Acitivity	Conversion (%)	Yield (%)
	(<i>R</i>) = 99	6	82	40
	(<i>S</i>) > 99	100	100	81
	(<i>R</i>) > 99	70	100	81
	(<i>R</i>) > 99	60	81	58
	(<i>S</i>) > 99	29	100	79
	(<i>S</i>) > 99	67	100	91
	(<i>R</i>) 79	2	12	8
	(<i>R</i>) > 99	17	79	17
	(<i>R</i>) > 99	104	83	31
	(<i>R</i>) > 99	229	87	38
	(<i>S</i>) > 99	809	100	54
	(<i>R</i>) > 99	4	35	24



SCHEME 32.4

that no expensive external cofactor might be used. In some instances, however, the application of an additional cofactor proved to be useful to increase the reaction rate.

In an early work, Matsumura et al. [44] demonstrated the use of *Candida boidinii* wild-type cells grown on methanol in the production of (*R*)-1,3-butandiol. They could recycle the cells several times; however, extremely high biocatalyst/substrate ratios (5 g vs. 100 mg) were applied, limiting the technical potential of this system. Instead of methanol, glucose could also be used as a regenerating system, leading, however, to much poorer results with respect to yield and ee.

Itoh et al. [45] found and recombinantly expressed a phenylacetaldehyde reductase from a *Corynebacterium* strain. The enzyme has 29% identity with the ADH from *Sulfolobus solfataricus* and 27% identity with the *C. parapsilosis* ADH. As can be seen from Table 32.5, the enzyme has broad substrate specificity.

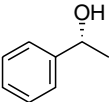
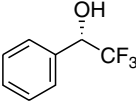
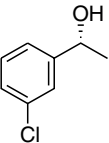
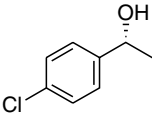
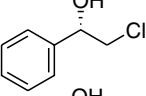
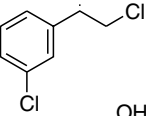
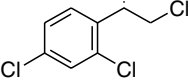
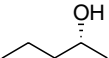
As a model, the conversion of α ,*m*-dichloroacetophenone was analyzed in more detail. The internal NADH-pool of the recombinant *E. coli* cells was insufficient to get high reaction rates. Therefore, 1 mM of NADH was added. The optimal 2-propanol concentration was 3 to 7%. The reaction rate was low, probably due to the inactivation of the enzyme. For all substrates analyzed, a very high enantioselectivity has been observed (Table 32.6).

For NADH regeneration, several secondary alcohols were tested at 5% v/v, including 2-propanol (relative activity at 3 mM: 6%), 2-butanol (15%), 2-pentanol (100%), 2-hexanol (240%), 2-heptanol (410%), and 2-octanol (422%). In the integrated reaction system, however, 2-propanol gave the highest conversion followed by 2-heptanol, 2-octanol, and 2-butanol.

In a major screening program, Matsuyama et al. [46] from Daicel found *C. parapsilosis* IFO 1396 exhibiting significant alcohol oxidizing activity when using (*R*)-1,3-butandiol as substrate. They also demonstrated that the enzyme could also be applied in the reductive mode using 2-propanol as reducing agent and without the addition of an external cofactor. They designated the enzyme as CpSADH. By recombinant expression of the enzyme in *E. coli* they were able to increase its activity by a factor of 78-fold. This recombinant biocatalyst reduces ethyl 4-chloroacetoacetate to ethyl (*R*)-4-chloro-3-hydroxybutanoate [(*R*)-ECHB] at 36.6 g/L with a yield of 95.2% and 99% ee (Scheme 32.5). (*R*)-ECHB is an important intermediate for the preparation of HMG-CoA-reductase inhibitors, a class of compounds that lowers the cholesterol level in human blood.

Amidjojo and Weuster-Botz [47] reported on the production of the corresponding product (*S*)-ECHB using *L. kefir* wild-type cells (analog to Scheme 32.5). The LKADH uses NADPH as cofactor, and again no additional cofactor was needed. The reaction was performed at high substrate concentration, which led to a two-phase system. With 5% (v/v) 2-propanol as cosubstrate, a final product concentration of 1.2 M, a yield of 97%, and an ee of 99.5% were achieved. The space-time yield was 85.7 mmol/L/h. Instead of 2-propanol, glucose can also be used as cosubstrate; however, the performance is much poorer.

TABLE 32.5

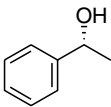
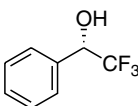
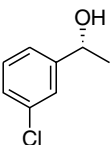
Substrate	Relative Rate of Reaction (%)
	100
	546
	258
	70
	188
	449
	868
	2247

Müller et al. [48] reported that by applying a recombinant *E. coli* strain expressing *L. brevis* ADH (LBADH) 3,5-dioxocarboxylates **1** can be reduced to the corresponding 5-hydroxy-3-oxocarboxylates **2** in a highly regio- and enantioselective manner, leaving the 3-oxo-group untouched (Scheme 32.6). Thus, with 2-propanol as reducing agent **1** is reduced to **2** with 99.4% ee and complete regioselectivity. The corresponding chloro-derivative **3** is converted with >99.5% ee to the expected *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate **4** (72% isolated yield).

Applying a substrate-coupled cofactor regeneration, the limited stabilities of the known ADHs against organic reducing agents like 2-propanol were a significant hurdle for the broader technical application as high cosubstrate concentrations are needed in order to get high conversion rates. Two ways to overcome these limitations have been demonstrated: applying a clever reaction design and screening for a solvent-tolerant enzyme.

During the reduction of ethyl 5-oxohexanoate (Scheme 32.7), high acetone concentration led to reducing the equilibrium concentration of ethyl (*S*)-5-hydroxyhexanoate. Liese et al.

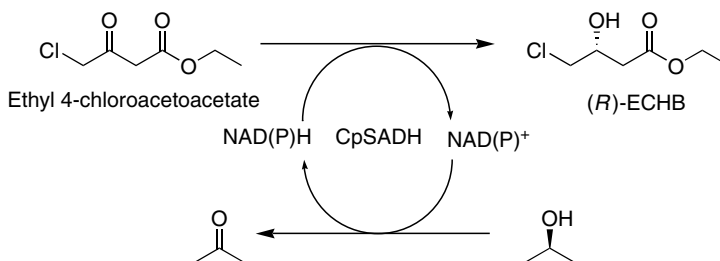
TABLE 32.6

Product	Enantiomeric Excess (%)
	$R \gg 99$
	$S \gg 99$
	$S \gg 99$

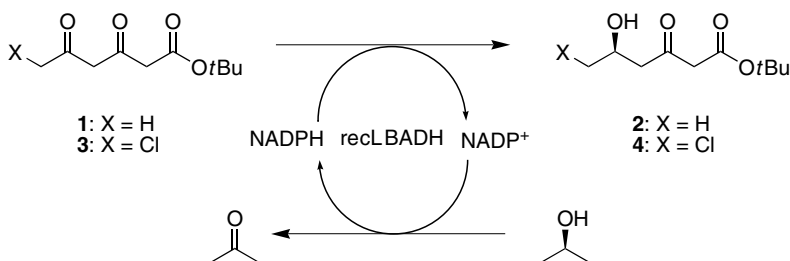
[31] demonstrated that by pervaporation or stripping of the acetone the conversion can be increased from 75 to 95 and >97%, respectively.

The Faber group reported conversion of ketones very selectively and efficiently to the corresponding (*S*)-alcohol by *Rhodococcus ruber* DSM 4451 at 2-propanol concentrations of up to 50% (v/v) and acetone concentrations of up to 15% (v/v) [32,49]. Thus, the flexibility in applying substrate-coupled cofactor regeneration was significantly increased. Specifically, for commercial applications the high 2-propanol tolerance of the strain and enzyme considerably broadened the scope of this reaction scheme and thus represented a major breakthrough. From *R. ruber* at least four NAD^+/NADH -dependent active fractions could be isolated which showed the same enantioselectivity. However, only one showed the pronounced stability toward 2-propanol and acetone and could use 2-propanol as a substrate. As can be seen from Table 32.7, many methyl alkyl ketones and methyl aryl ketones are converted with an enantioselectivity of more than 99%. Shifting the keto-function to the 3-position, however, resulted in a drop of the enantioselectivity to 97%.

The ADH from *R. ruber* not only exhibits a high solvent stability but is also stable at temperatures up to 60°C and active over a broad pH range from 5.5 up to 11 [50]. Meanwhile, the Faber group also reported the recombinant expression of the *R. ruber* ADH, thus further expanding the scope of this versatile enzyme [51].



SCHEME 32.5



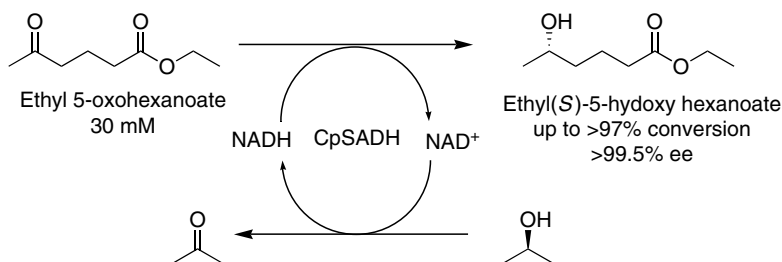
SCHEME 32.6

32.4 PROCESSES BASED ON ENZYME-COUPLED COFACTOR REGENERATION WITH A FORMATE DEHYDROGENASE

32.4.1 THE CONCEPT

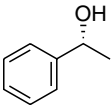
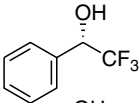
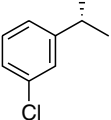
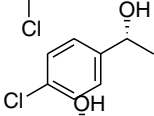
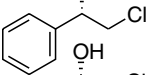
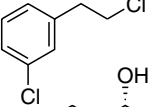
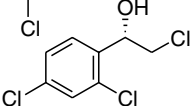
When applying enzyme-coupled cofactor regeneration for asymmetric biocatalytic reduction processes, the use of an FDH turned out to represent a very popular approach. The FDH catalyzes the oxidation of formate to carbon dioxide, while reducing the oxidized form of the cofactor into its reduced form, NAD(P)H. The whole concept of an asymmetric reduction of ketones in the presence of an ADH and FDH is shown in [Scheme 32.8](#).

The most widely applied FDH is probably the FDH from *C. boidinii* and optimized mutants thereof [52], developed in the Kula group, who are—jointly with Wandrey et al.—the pioneers in the field of FDH-based cofactor regeneration [53,54], as well as the Whitesides group [55]. Although limited to the regeneration of NADH alone, the FDH from *C. boidinii* is available in recombinant form on a large scale, and has already proved its suitability for industrial purpose in the reductive amination of trimethylpyruvate, a process which is running on industrial scale at Degussa AG for the manufacture of *L-tert-leucine* [56,57]. In addition to the FDH from *C. boidinii*, there are further FDHs known to be suitable for biotransformation such as that from the methylotrophic bacterium *Pseudomonas* sp. [58] and from *Mycobacterium vaccae* N10 [59,60]. A key advantage when using an FDH for cofactor regeneration certainly is the irreversible step of carbon dioxide formation and removal, thus shifting the equilibrium toward (complete) product formation. However, not only is the equilibrium shifted toward the product side, leading to high conversions, but the downstream processing is also simplified as (ideally) no organic by-product is generated in the reaction mixture. A general limitation of FDHs so far is the specific activity that does not exceed the range of 10 U/mg in spite of numerous attempts for improvement. For example, the FDH from *C. boidinii* shows a specific activity of about 6 U/mg. Thus, these activities are low in

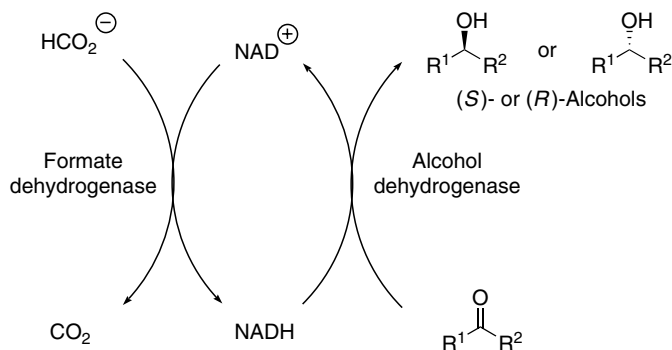


SCHEME 32.7

TABLE 32.7

Substrate	Enantiomeric Excess (Product) (%)	Conversion (%)
	>99	81
	>99	82
	>99	92
	>99	91
	97	79
	>99	70
	> 99	65

comparison with those for related cofactor-regenerating enzymes such as the GDH and malate dehydrogenase, which typically show activities of >100 U/mg. However, as can be seen from the subsequent examples, the FDH is an efficient and highly suitable enzyme for cofactor regeneration in synthetic reduction processes in spite of its limitations.



SCHEME 32.8

32.4.2 PROCESSES WITH ISOLATED ENZYMES

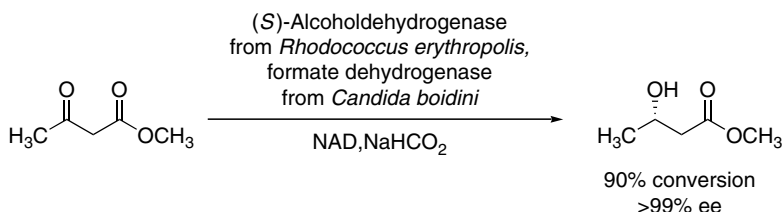
The initial work on the enzymatic reduction of ketones has been carried out based on the use of isolated enzymes in homogeneous aqueous media. Due to the low solubility of the hydrophobic ketones in water, the reactions were carried out at low substrate concentrations for a long time, typically in the range of 5 to 20 mM or below. In 1993, Hummel and Gottwald reported the suitability of an ADH from *R. erythropolis* in combination with an FDH for asymmetric reduction of ketones at a substrate concentration of 1.16 mM [61]. In addition, the Kula and Wandrey groups studied in detail this cofactor regeneration methodology for the preparation of a variety of alcohols using ADHs from *R. erythropolis* [62], *C. parapsilosis* [63], and *C. boidinii*, respectively. Therein, the NADH-dependent ADH from *R. erythropolis* turned out to be stable at 37°C for a couple of days, and suitable for the reduction of a broad variety of ketones comprising keto esters, aromatic ketones, and aliphatic 2-alkanones [62]. This enzyme shows both a high enantioselectivity and specific activity with, for example, 269 U/mg for ethyl acetoacetate. However, a key feature of most highly suitable substrates is the presence of an aceto-moiety, as in case of most of the ADHs reported so far.

Adlercreutz reported a crude enzyme preparation from permeabilized and lyophilized *Gluconobacter oxydans* cells to contain suitable ADH(s) for enantioselective reduction of a variety of ketones with high enantioselectivity of 93 to 99% in most cases [64].

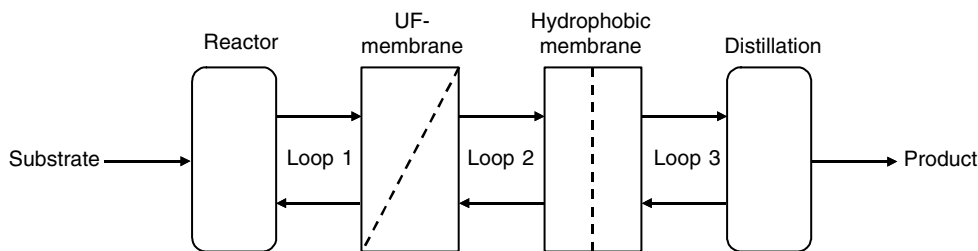
Another example for the suitability of the FDH-based cofactor regeneration concept for asymmetric ketone reduction has been demonstrated by the Itoh group using an (*S*)-selective ADH isolated from styrene-assimilating *Corynebacterium* strain ST-10 [65]. An impressive range of ketones, comprising alkanones and aromatic ketones, has been reduced with excellent enantioselectivities of >99% in many cases. So far, however, reactions were carried out at a low substrate concentration of <10 mM, often <1 mM.

The Kula group also carried out preparative conversions based on these enzymes by coupling the ADH reduction reactions with the FDH regeneration (Scheme 32.9) [66,67]. As enzymes, ADHs from *R. erythropolis* and *C. parapsilosis* were used in combination with the FDH from *C. boidinii*. Carrying out the reductions of several keto esters and a keto dialkyl acetal at a substrate concentration of 100 mM in most cases furnished the desired alcohols with high conversion (up to 100%), and high enantioselectivities of >99%. A selected example is given in Scheme 32.9 [66].

A modified methodology for enantioselective ADH- and FDH-coupled reductions in aqueous media has also been reported by Wandrey et al. carrying out enzymatic reductions in cyclodextrin-containing buffers [68]. The corresponding alcohols were obtained in good yield and with high enantioselectivities when using an ADH from *C. parapsilosis*. Notably, high stability of the enzymes in media containing heptakis-(2,6-di-O-methyl)- β -cyclodextrin has been found. In the continuous reaction with the ADH and an FDH from *C. boidinii*, a steady conversion of up to 79% for the synthesis of (*S*)-1-(2-naphthyl)ethanol has been achieved.



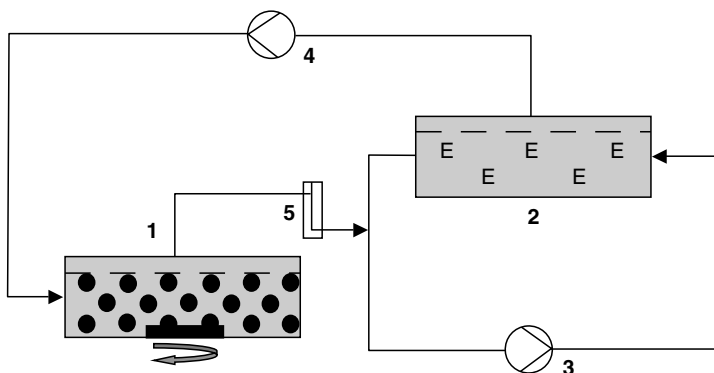
SCHEME 32.9



SCHEME 32.10

Also the issue of high space–time yields in spite of the limitation of low ketone solubility has been successfully addressed by the Wandrey group, who developed a very elegant engineering solution by means of a continuous process with an enzyme-membrane reactor (Scheme 32.10). An efficient “three-loops”-concept is based on an enzymatic reaction in pure aqueous medium, a separation of the aqueous phase from the enzyme by ultrafiltration, and a subsequent continuous extraction of the aqueous phase with an organic solvent. Organic and aqueous phases are separated by a hydrophobic membrane [69–71]. This is required as organic solvents can generally cause significant enzyme inactivation. In particular, this is known for the FDH from *C. boidinii* which is sensitive to organic solvents [72]. Although the reaction in this enzyme-membrane reactor is limited by the low solubility of the ketone in water (9 to 12 mM), good space–time yields in the range of 60 to 104 g/(L/d) have been obtained [69–71]. With respect to synthesized products, for example, (*S*)-1-phenylpropan-2-ol and (*S*)-4-phenylbutan-2-ol have been produced in enantiomerically pure form. Typical data for the consumption of the enzymes are ~3.0 to 3.5 kU/kg for the ADH and ~5 to 10 kU/kg for the FDH.

An extended, newly designed emulsion-membrane reactor concept has been successfully applied by Wandrey et al. for the asymmetric reduction of 2-octanone [73]. As enzymes, the ADH from *C. parapsilosis* (CP-ADH) and an FDH from *C. boidinii* have been used. The reactor consists of a stirred emulsion vessel, from which only the aqueous phase—separated by a hydrophilic ultrafiltration membrane—enters the enzyme-membrane reactor (Scheme 32.11).



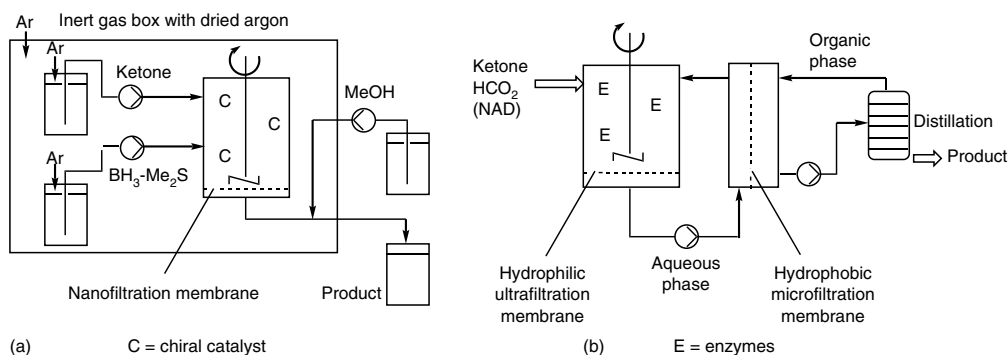
- 1: Stirred emulsion vessel with hydrophilic ultrafiltration membrane.
 2: Enzyme-membrane reactor loop with ultrafiltration module.
 3,4: Circulation pumps. 5: Bubble trap; E: Enzyme.

SCHEME 32.11

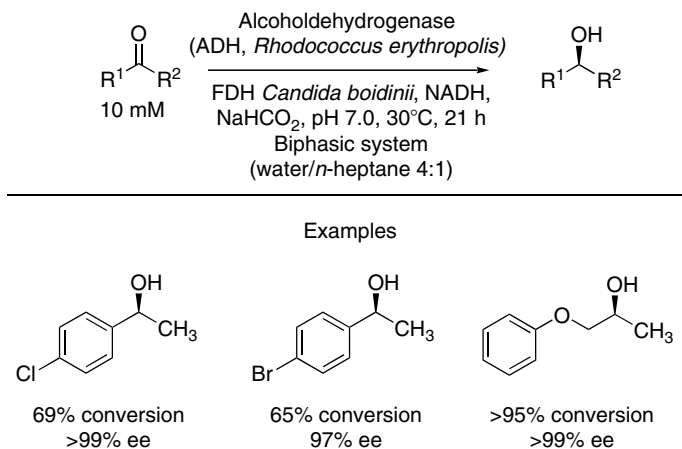
Therein, the desired enzymatic reduction takes place at a pH of 7, and the product outflow is recirculated to the stirred emulsion reactor, where the alcohol is extracted into the organic phase, and the water phase is recharged with substrate. A conversion of 87% (with a residence time of 1 h) has been achieved over a period of 200 h. The conversion itself has been further increased to 97% at a residence time of 1 h when increasing the formate concentration from 12 to 50 mM, corresponding to a space–time yield of 21.1 g/(L/d). Excellent half-life times have been found with $\tau_{1/2} = 67$ d for the CP-ADH and $\tau_{1/2} = 88$ d for the FDH. Compared with the “classic” enzyme-membrane reactor the total turnover number has been increased by factor 9. Thus, this emulsion-membrane reactor, which has been operated over a period of >4 months, overcomes limitations of large volumes and low total turnover numbers.

An interesting comparative study of the ADH- and FDH-based reduction with an oxazaborolidine-catalyzed borane reduction has been done by the Wandrey group [74]. Both reactions were carried out in continuously operating membrane reactors, which are shown in Scheme 32.12. Notably, the stability of the enzymes is higher with a half-life time of 31.1 d compared with 1.2 d for the chemical catalyst. Furthermore, enantioselectivity of the enzymatic reaction was >99% ee, whereas a somewhat lower enantioselectivity of 90 to 94% ee was obtained in the chemical reduction method. However, space–time yield of the chemical approach, which takes place in an organic solvent, was higher with 1.4 kg/(L/d) compared with 0.088 kg/(L/d) for the enzymatic approach. The lower productivity when using enzymes has been explained with the low solubility of substrate and product in water.

The search for suitable reaction media, which guarantee high ketone solubility, however, has been ongoing. Although the presence of an organic solvent could improve the solubility of poorly water-soluble ketones, the known instability of the FDH from *C. boidinii* toward many organic solvents remains a challenge. Addressing this issue, Gröger et al. developed a suitable aqueous–organic two-phase solvent reaction medium based on the use of *n*-heptane and *n*-hexane as organic phases (Scheme 32.13) [75,76]. This reaction medium fulfils the criteria that both enzymes, namely the recombinant (*S*)-ADH from *R. erythropolis* and FDH from *C. boidinii* (and mutants thereof), remain stable in the presence of the organic solvent. In addition, a good solubility of poorly water-soluble ketones led to substrate concentrations of up to 200 mM with a simple reaction protocol. In contrast, the stability of the FDH from *C. boidinii* (mutant C23S, C262A) [53] was low in the presence of only 10% (v/v) of many organic “standard” solvents such as MTBE, ethyl acetate, and toluene. With this enzyme-compatible reaction medium at hand, preparative conversions were carried out that gave good conversions accompanied by high enantioselectivities with a variety of aromatic ketone substrates (Scheme 32.13).



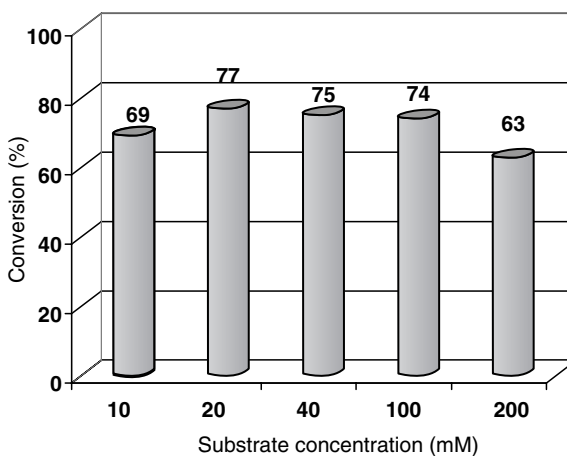
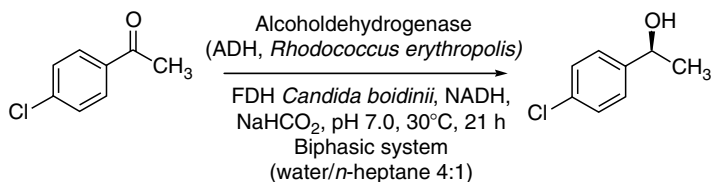
SCHEME 32.12



SCHEME 32.13

For example, in the presence of the (*S*)-ADH from *R. erythropolis*, *p*-chloroacetophenone was converted to the optically active (*S*)-enantiomer with >99% ee and a conversion of 69%. Furthermore, the reactions also proceed in water–*n*-heptane two-phase reaction media at higher substrate concentrations of up to 200 mM (Scheme 32.14) [75,76]. However, at >200 mM substrate concentration, lower conversions were obtained and prolonged reaction times were required.

A further improvement of the substrate concentrations up to 500 mM has been realized when using an “emulsion system” for the synthesis of the corresponding alcohols [77,78].



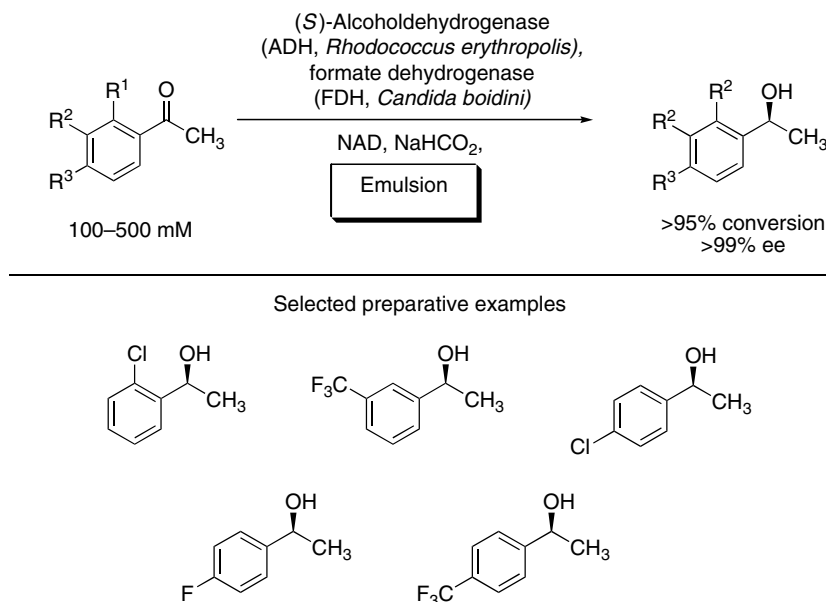
SCHEME 32.14

When carrying out the reductions with an ADH and FDH in pure aqueous media, both high conversions and high enantioselectivities were obtained at substrate concentrations of up to 500 mM (Scheme 32.15). For example, the reduction of 4-chloroacetophenone as a model substrate on a 6 L scale gave the desired (*S*)-alcohol with >98% conversion and >99.4% ee. As enzymes, the ADH from *R. erythropolis* and the FDH from *C. boidinii* have been used.

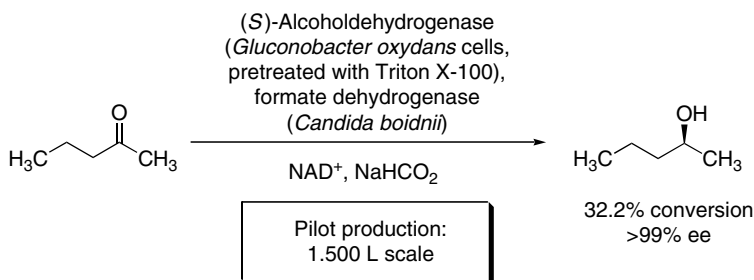
Furthermore, ADH- and FDH-based reduction processes have already proved their technical feasibility. This has been successfully demonstrated by the Patel group in the production of (*S*)-2-pentanol on pilot scale using an ADH from *G. oxydans* (SC 13851) [79]. As biocatalyst, *G. oxydans* cells pretreated with Tritone X-100 were used (after a freeze/thaw cycle) in combination with the FDH from *C. boidinii*. The latter enzyme was used in a partially purified form in order to prevent undesired formation of racemic product, which might be due to the presence of other dehydrogenases in the crude extract. Notably, the reduction was carried out at a 1.500 L scale. After a reaction time of 46 h and at a substrate input of 3.2 kg (~2.13 g/L), the desired (*S*)-(+)-2-pentanol was formed with a conversion of 32.2% and an enantioselectivity of >99% (Scheme 32.16).

Another elegant and efficient approach in process design has been reported by the Schomäcker group [80]. Therein, an enzymatic reduction with FDH-based NADH regeneration of a less water-soluble ketone was carried out in reverse micelles. When using these types of water-in-oil microemulsions as reaction media, the reduction of 2-heptanone under formation of (*S*)-2-heptanol proceeds with complete conversion and an excellent enantioselectivity of >99%. Furthermore, it turned out that the reaction rate of ADH in microemulsions increases up to 12 times compared with the rate in water. The concept of this microemulsion reaction system is shown in Scheme 32.17.

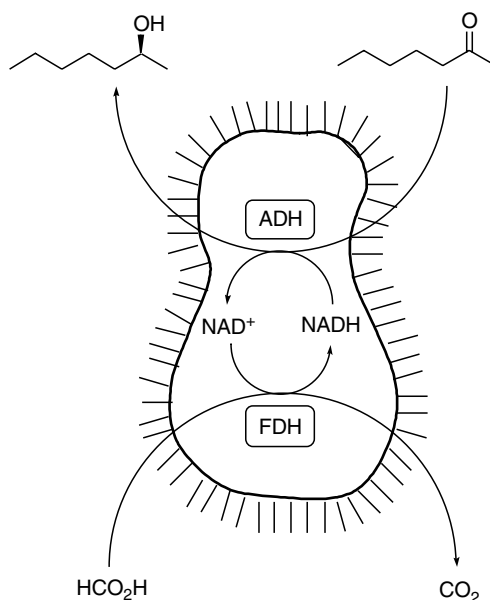
A further study by the same group focusing on the stability and activity of different types of ADHs in water-in-oil microemulsions showed significant changes of activity and stability of these enzymes depending on the water and surfactant concentration of the microemulsion, whereas—notably—the FDH from *C. boidinii* did not show any kinetic effect depending on the microemulsion components [81].



SCHEME 32.15



SCHEME 32.16

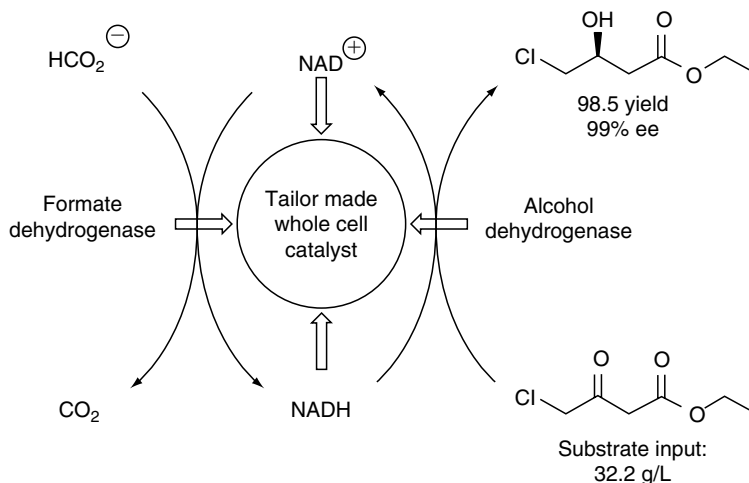


SCHEME 32.17

32.4.3 WHOLE-CELL REDUCTIONS

The potential of an FDH-based whole-cell catalyst for synthetic applications has been reported by Matsuyama et al., using a recombinant *E. coli* W3110 strain, which coexpresses an ADH from *Pichia finlandica* and an FDH from *Mycobacterium* [82]. As motivation for using the FDH, the authors mentioned the low affinity of the ADH for 2-propanol as a substrate, thus making the substrate-coupled cofactor regeneration method not attractive in this case. A “one-plasmid-strategy” has been used inserting the ADH- and FDH-genes in a single plasmid. The tailor-made whole-cell catalyst has subsequently been applied in the enantioselective reduction of ethyl 4-chloro-3-oxobutanoate to the corresponding (*S*)-alcohol at 32.2 g/L, with 98.5% yield and 99% ee (Scheme 32.18). A further successful example is the synthesis of (*R*)-1-chloro-4-pentanol from the corresponding ketone at 26.1 g/L with 99% ee.

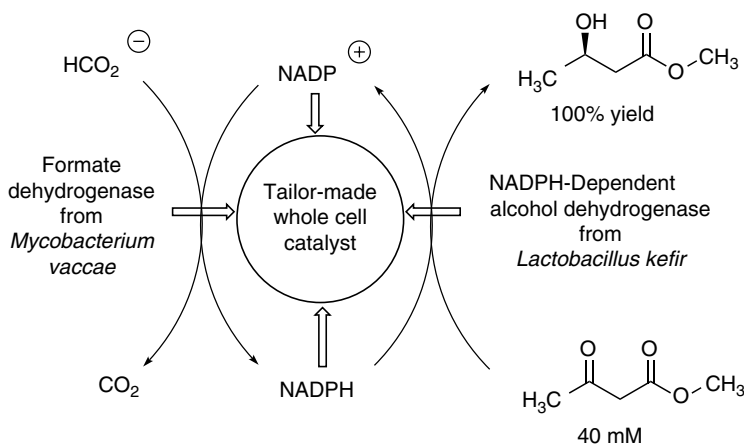
Another ADH- and FDH-based whole-cell system, which contains an NADPH-dependent ADH from *L. brevis* with the FDH from *M. vaccae* N10, has been constructed by the Sahm group [83]. The FDH from *M. vaccae* N10, is suitable for the regeneration of both NADH and NADPH. Overexpression of both enzymes, ADH and FDH, led to an efficient



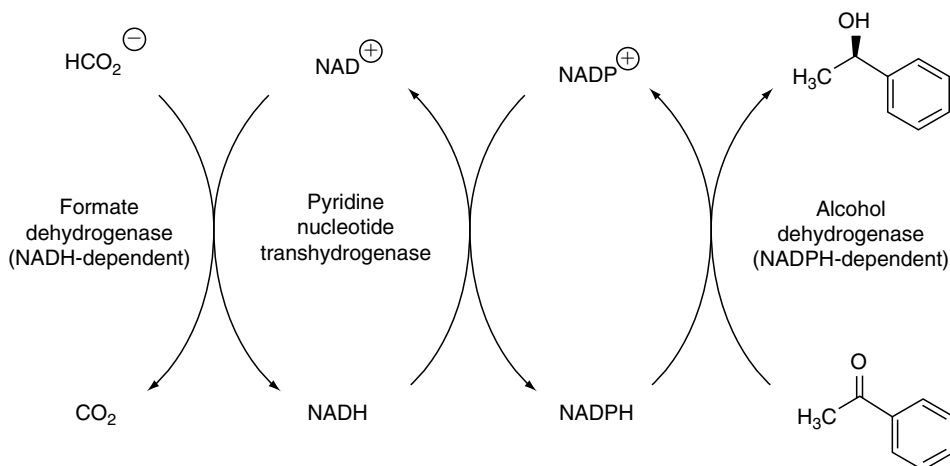
SCHEME 32.18

whole-cell biocatalyst, which gave a quantitative conversion in the reduction of methyl acetoacetate at a substrate concentration of 40 mM (Scheme 32.19). It is noteworthy that the presence of the FDH led to a sevenfold increase of the intracellular NADH/NAD^+ ratio in the recombinant cells.

For a long time, a major limitation for applications using the FDH from *C. boidinii* was its inability to regenerate NADP^+ , thus being limited to the regeneration of NAD^+ only. An elegant solution for this problem has been recently found by the Hummel group, thus expanding the application range of this FDH-based cofactor regeneration also to NADP^+ -dependent ADHs [84]. As an ADH, the ADH from *L. kefir* [85,86] was chosen. The key step is the integration of an additional enzymatic step within the cofactor regeneration cycle, namely the pyridine nucleotide transhydrogenase (PNT)-catalyzed regeneration of NADPH from NADP^+ under consumption of NADH forming NAD^+ [84]. The concept is graphically shown in Scheme 32.20, exemplified for the synthesis of phenylethanol. The Hummel group constructed a whole-cell system, coexpressing the ADH from *L. kefir*, PNT from *E. coli*, and



SCHEME 32.19



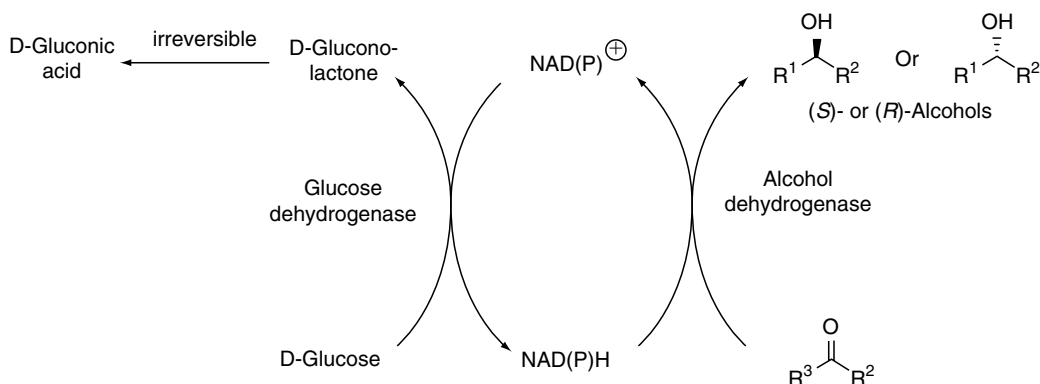
SCHEME 32.20

FDH from *C. boidinii*. In the presence of this catalyst, the desired product was formed in 66% yield at a substrate concentration of 10 mM in the presence of both cofactors.

32.5 PROCESSES BASED ON ENZYME-COUPLED COFACTOR REGENERATION USING A GLUCOSE DEHYDROGENASE

32.5.1 THE CONCEPT

An efficient option for recycling the cofactor NADH is based on the use of a GDH. Therein, glucose is oxidized to the corresponding gluconolactone, while the oxidized cofactor NAD(P)^+ is reduced to NAD(P)H required as reducing agent for the reduction process. This concept of cofactor regeneration is shown in Scheme 32.21. As the gluconate is subsequently hydrolyzed to gluconic acid (as its sodium salt at neutral pH), this reaction also can be regarded as an irreversible step, thus shifting the whole reaction into the direction of the desired alcohol product. Compared to the FDHs described in [Section 32.4](#), the GDHs offer the advantage of higher specific activities, which are typically >100 U/mg (compared with FDH activity of ~ 10 U/mg).



SCHEME 32.21

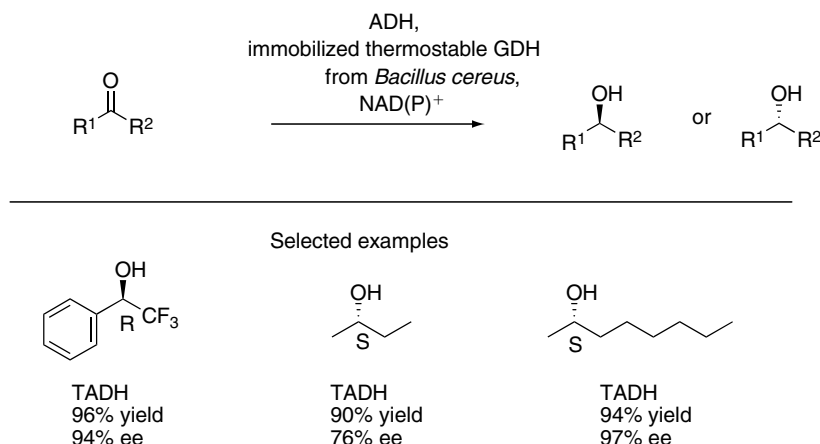
In addition, numerous GDHs are already known and available in recombinant form, which is a prerequisite for an efficient application on technical scale. Typical examples are the GDH from *Bacillus megaterium* [87,88], *B. subtilis* [89,90], *B. cereus* [91,92], and *Thermoplasma acidophilum* [93]. Although some preparative synthetic applications by means of isolated enzymes are known, most of the reported applications of GDH-coupled cofactor regeneration in asymmetric reduction are based on the use of recombinant whole-cell systems. Notably, industrial applications of this recombinant whole-cell technology based on an ADH and a GDH have already been reported by specialty chemicals companies, in particular by Kaneka Corporation and Degussa AG.

32.5.2 PROCESSES WITH ISOLATED ENZYMES

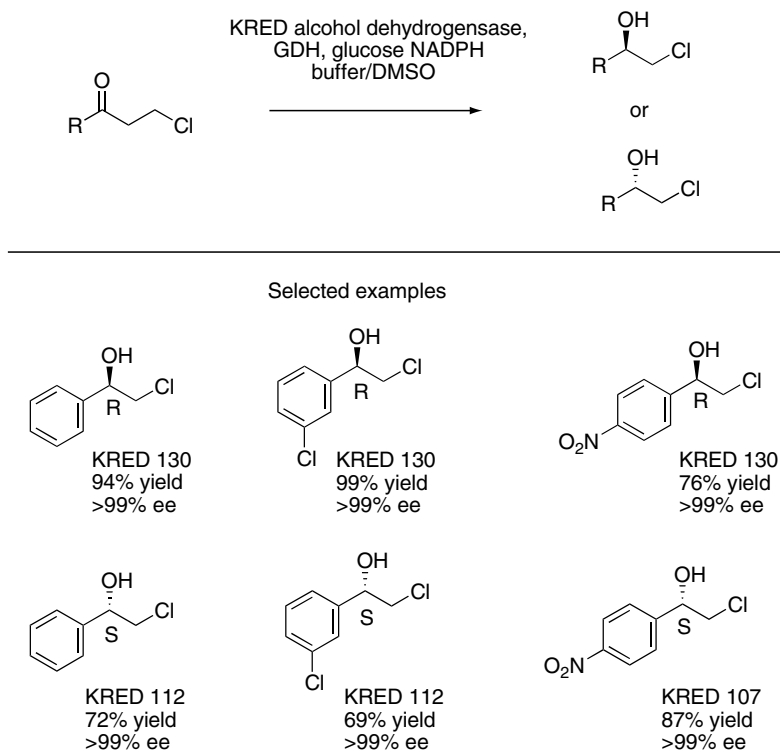
The proof of principle and pioneering work for a biocatalytic reduction using a GDH-coupled cofactor regeneration process has been done by Wong et al., who also described a readable overview of the motivation to use this type of enzymes [94,95]. Notably, the GDH from *B. cereus* is a thermostable enzyme and is beneficial in terms of stability. In addition, it has a high specific activity of 250 U/mg. The corresponding enzymatic reduction of ketones in the presence of different types of ADHs, such as ADHs from horse liver, yeast, and *T. Brockii* (TADH), gave the desired alcohols with good to high enantioselectivities. Both ADH and GDH were used in an immobilized form. The conversions of these enzymatic biotransformations were in the range of 72 to 90%. Although enantioselectivities varied, they exceeded 90% ee in many cases. Selected examples are shown in Scheme 32.22. The reactions are typically carried out at a substrate concentration of 125 mM.

This reduction method based on the use of isolated ADH and GDH enzymes has also been applied for the synthesis of (*S*)-sulcatol [96]. In the presence of the NADPH-dependent ADH of *T. Brockii* and at a substrate concentration of 25 mM, sulcatone was reduced with a conversion of about 84%. Compared with the 2-propanol-based cofactor regeneration approach (conversion: 100%), conversion was somewhat lower when using a GDH.

A recent contribution to this field has been made by the Hua group focusing on the asymmetric reduction of α -chlorinated ketones in the presence of isolated ADHs and under regeneration of the cofactor with a GDH [97]. The reactions have been carried out at a substrate concentration of ~ 4 g/L, and led to the formation of a range of α -chlorinated



SCHEME 32.22

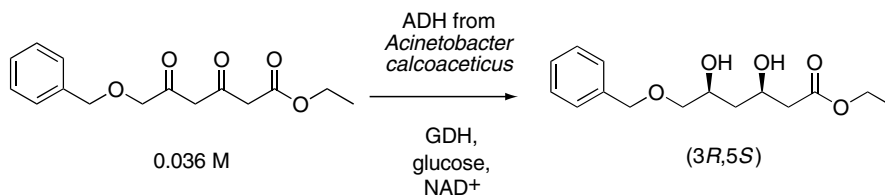


SCHEME 32.23

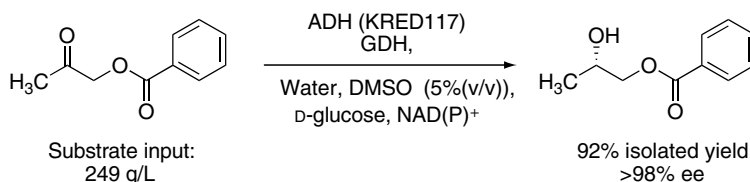
alcohols in high yields of 72 to 99%, with excellent enantioselectivities of typically >99% ee. Selected examples are shown in Scheme 32.23.

The feasibility of this methodology for an enantio- and diastereoselective reduction of a ketone, namely ethyl 6-benzyloxy-3,5-dioxohexanoate, has been demonstrated by the Patel group [98,99]. When using cell extracts of *Acinetobacter calcoaceticus* in combination with a GDH and glucose, the desired product ethyl (3*R*,5*S*)-6-benzyloxy-3,5-dihydroxyhexanoate was obtained with a conversion of 92% and an ee of 99% (Scheme 32.24). After product isolation, (3*R*,5*S*)-6-benzyloxy-3,5-dihydroxyhexanoate was obtained in 72% yield, with an ee of 99.5%.

In addition to screening for various ketones, the preparative asymmetric reduction of benzoyl hydroxyacetone and α -tetralone in the presence of isolated ADH and GDH enzymes has been described by BioCatalytics researchers [100]. Using the isolated ADH enzymes in an amount of 1 to 7% (w/w) compared with the amount of substrate and a catalytic amount of cofactor led to the synthesis of the optically active alcohols in high yields. Reductions have



SCHEME 32.24



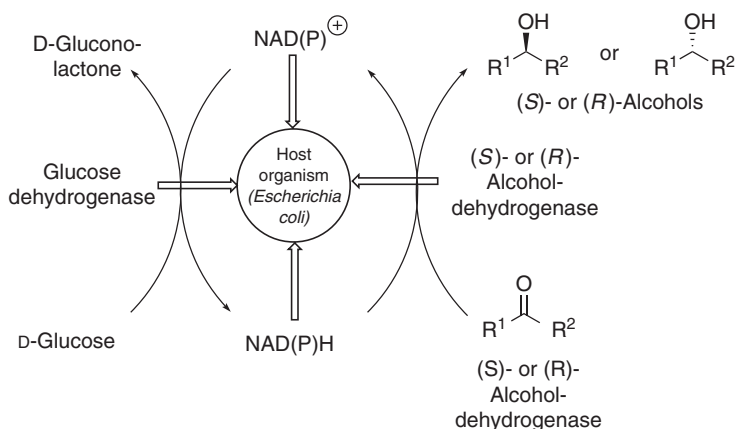
SCHEME 32.25

been carried out at high substrate concentrations of up to 0.75 to 1.4 M. A selected example is shown in Scheme 32.25.

32.5.3 WHOLE-CELL REDUCTIONS

The design of recombinant whole cells is an elegant approach toward tailor-made (bio-)catalysts, called “designer bugs,” which contain not only the cofactor “for free” but also both of the desired enzymes, ADH and GDH, in overexpressed form. The corresponding reduction of ketones proceeds within the cell according to the concept shown in Scheme 32.26, thus converting glucose in a single step into gluconolactone, which itself is subsequently opened into its free acid form, gluconic acid. In parallel, the cell-internal ADH consumes the formed NAD(P)H by reducing the prochiral ketone into the desired optically active alcohol. Advantages of such a recombinant whole-cell system over the wild-type ones are the higher amount of the desired enzymes within the cell (due to overexpression) and its ability to produce these biocatalysts in a high-cell density fermentation process. Therein, wet biomass concentrations of >200 g/L of fermentation broth can be obtained, thus representing a cost-effective approach toward recombinant whole-cell catalysts. In addition, performance of the cells with respect to enantioselectivity is better in the case of recombinant whole cells, as only the desired enzymes, namely ADH and GDH, are produced in large amounts. In contrast, wild-type organisms contain a multitude of ADHs, which compete for the corresponding substrate. Due to the presence of substrate-tolerating (*R*)- and (*S*)-selective ADHs this often leads to low, nonsatisfactory enantioselectivities.

As synthetic efficiency depends on the performance of the (bio-)catalyst, the construction of these catalysts is an issue of crucial importance for the later process. A well-known approach is based on the use of *E. coli* as a host organism suitable for high cell density fermentation and, thus, economical production.

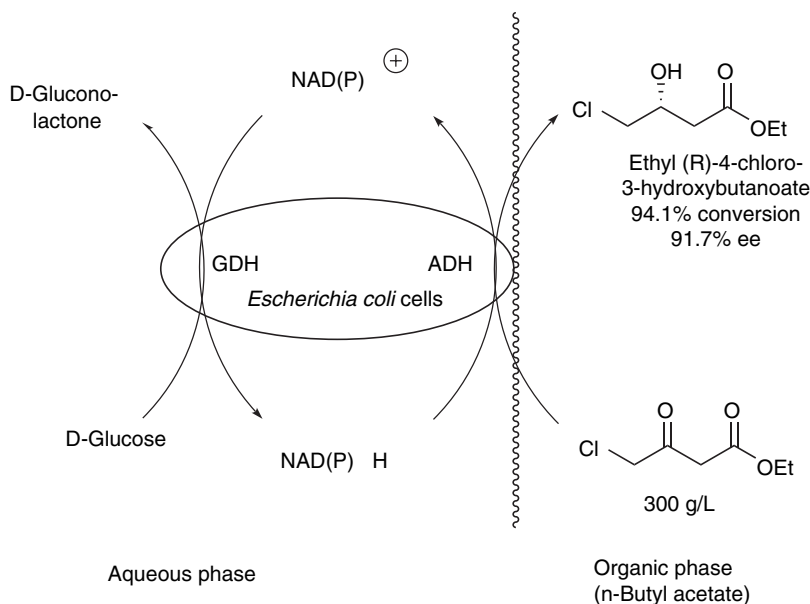


SCHEME 32.26

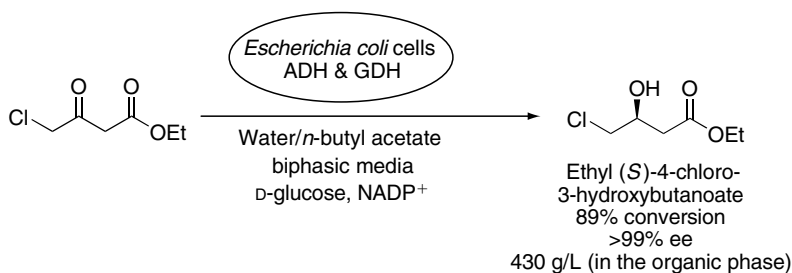
With respect to this recombinant whole-cell concept, the pioneers in the design and application of highly efficient recombinant whole-cell biocatalysts, consisting of an ADH and GDH, are Shimizu et al. [101]. As a GDH, the GDH from *B. megaterium*, which accepts both NADH and NADPH as a cofactor, was used. Already in the 1990s, Shimizu et al. had developed an effective *E. coli* catalyst, as well as a highly efficient reaction system for the reduction of 4-chloro-3-oxobutanoate. To start with the developed recombinant ADHs, several enzymes from *Sporobolomyces salmonicolor* [102–104] and *C. magnoliae* [105,106] have been screened, characterized, and successfully cloned in *E. coli* together with the GDH gene. Thus, a library of highly efficient biocatalysts, namely *E. coli* transformant cells coexpressing the corresponding ADH and GDH from *B. megaterium*, has been successfully established by Shimizu et al. [101].

The use of these efficient recombinant whole-cell catalysts in the asymmetric reduction of 4-chloro-3-oxobutanoate forming the corresponding pharmaceutically important alcohol has been intensively investigated and optimized by the Shimizu group. As a reaction media, an *n*-butyl acetate–water two-phase solvent system turned out to be very suitable [107]. When using the *E. coli* host organism overexpressing an NADP⁺-dependent ADH from *S. salmonicolor*, and an isolated GDH enzyme or GDH-expressing cells as biocatalysts, the desired optically active (*R*)-alcohol was formed with up to 255 g/L in the organic phase under optimized conditions [108,109]. The conversion reached 91% and an ee of 91% was obtained [109]. In addition to glucose as a cosubstrate, a low amount of NADP⁺ is required. A further improvement has been achieved when using *E. coli*, coexpressing both the ADH from *S. salmonicolor* and the GDH from *B. megaterium*, resulting in the formation of the desired optically active (*R*)-alcohol with 94.1% conversion and 91.7% ee at a substrate concentration of 300 g/L in the presence of a catalytic amount of the NADP⁺ cofactor [110]. In Scheme 32.27, the concept of this application of a tailor-made whole-cell biocatalyst in a two-phase reaction media along with the experimental results are shown.

It is noteworthy that the Shimizu group also designed a whole-cell catalyst for the synthesis of the analog (*S*)-enantiomeric form of ethyl 4-chloro-3-hydroxybutanoate, which is pharmaceutically an important intermediate [111]. When using the recombinant *E. coli*



SCHEME 32.27



SCHEME 32.28

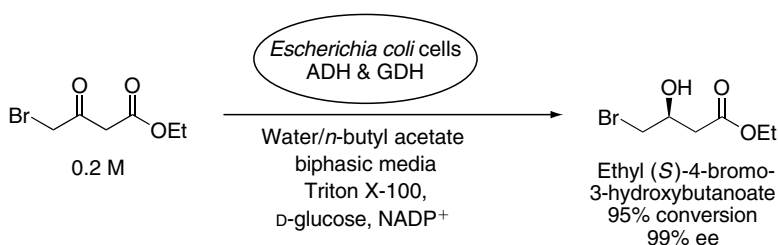
whole-cell catalyst, harboring an ADH from *C. magnoliae* and the GDH from *B. megaterium*, in an *n*-butyl acetate–water two-phase reaction media, the desired reduction proceeds with a conversion of up to 89%, leading to 430 g/L of product in the organic phase (Scheme 32.28). The ee was >99%, and the turnover number (TON) of NADP^+ was high, with a TON of up to 16,200. When feeding the substrate continuously, operating in aqueous solution has been possible at a substrate concentration of 208 g/L, leading to 96% conversion and >99% ee. With respect to the added NADP^+ , a TON of 21,600 was achieved.

This impressive technology developed by the Shimizu group has already been commercialized. Since 2000, Kaneka Corporation has applied this methodology to manufacture ethyl (S)-4-chloro-3-hydroxybutanoate on an industrial scale [101].

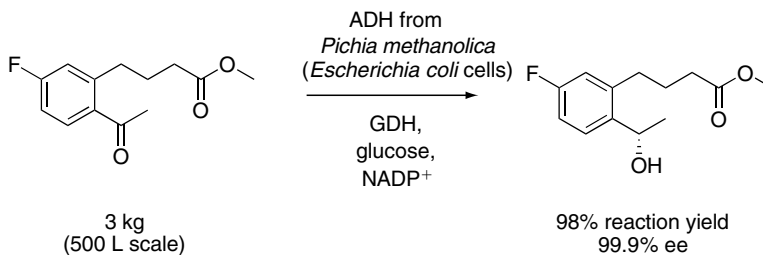
In addition, Kaneka researchers jointly with the Shimizu group reported the extension of this reduction technology for the synthesis of other types of functionalized β -keto esters [112]. For example, reduction of 4-bromo-3-oxobutanoate at a substrate concentration of 0.4 M in the presence of an *E. coli* biocatalyst proceeds with >95% conversion, reaching a concentration of 0.38 M in the organic phase, within 20 h. The reaction course is shown in Scheme 32.29. The reaction was carried out in a two-phase system using *n*-butyl acetate as an organic phase and a catalytic amount of the NADP^+ cofactor. In addition, the whole-cell reduction methodology has been further extended to a range of other substrates [101].

The application of a recombinant whole-cell catalyst, containing an ADH from *P. methanolicus* SC 13825, has been developed by Patel et al., and successfully applied for the reduction of an acetophenone substituted with a keto ester–containing moiety [113]. In this case, however, an “external” amount of GDH has been added. The reaction proceeded with a yield of 95% leading to an ee of 99.9% (Scheme 32.30). Notably, this biotransformation has been scaled up to a 500 L scale.

The construction of an *E. coli* whole-cell catalyst, harboring the widely used (*R*)-selective ADH from *L. kefir* and a GDH from *B. subtilis*, has been successfully completed by the Hummel group [114]. Interestingly, this GDH from *B. subtilis* also accepts both cofactors,



SCHEME 32.29



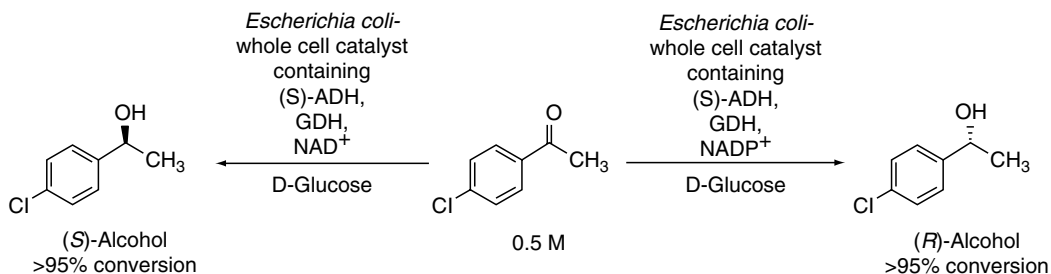
SCHEME 32.30

NAD⁺ and NADP⁺. Several reactions with acetophenone in many water–organic two-phase reaction media have been done using a substrate concentration of 20 mM. In general, the limited permeability of the membrane under the applied reaction conditions and limited stability of *E. coli* cells in organic solvents have been identified as limitations of the whole-cell approach.

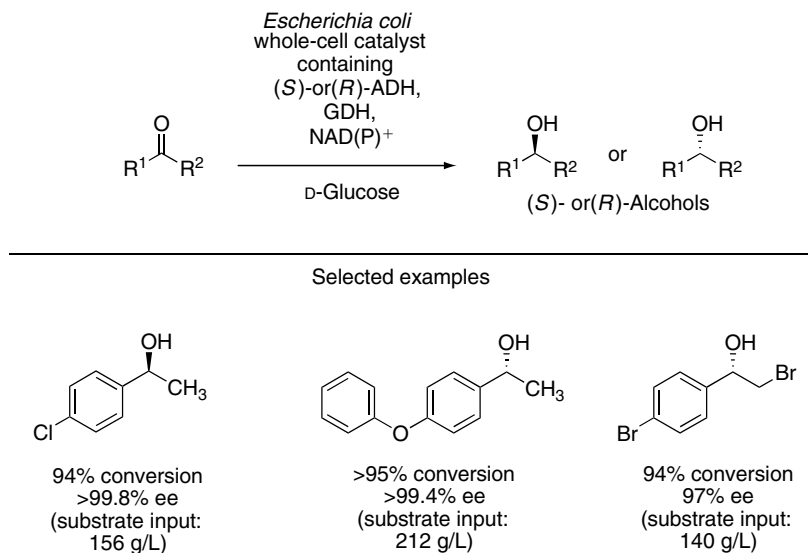
In addition, Degussa AG researchers jointly with the Hummel group developed recombinant whole-cell biocatalysts and applied them in asymmetric reductions of a range of ketones at high substrate input, exceeding 150 g/L, in pure aqueous media, in general without the need of an external amount of cofactor [115]; in some exceptional cases a very low amount of cofactor had to be added with respect to a sufficient reaction course. Both types of enantiomers are available due to the use of (*S*)- and (*R*)-selective whole-cell biocatalysts. While the latter is based on the use of an ADH from *L. kefir*, an ADH from *R. erythropolis* has been used for the former. The suitability of both of these biocatalysts has been demonstrated at first by the synthesis of both enantiomeric forms of 4-chlorophenylethan-1-ol at a substrate concentration of 0.5 M (Scheme 32.31).

Subsequently, this methodology, which is both economical and simple to be carried out, has been used for the preparation of a wide range of optically active alcohols. Typically, the substrate concentrations have been in the range of 0.5 to 1 M, thus exceeding 100 g/L. An overview of examples is given in Scheme 32.32. Notably, in all of these examples the external addition of a cofactor was not required. In the presence of the tailor-made (*R*)- or (*S*)-selective whole-cell catalyst, the reduction proceeds with high conversions of up to 95% and an ee of up to 99.4%.

Furthermore, a 10 L scale reduction of 4-chloroacetophenone as a model substrate gave the (*R*)-4-chlorophenylethan-1-ol in 95% conversion and >99.8% ee [115]. After further process development and scale up, this recombinant whole-cell reduction technology platform has been applied on an industrial scale at Degussa AG.



SCHEME 32.31



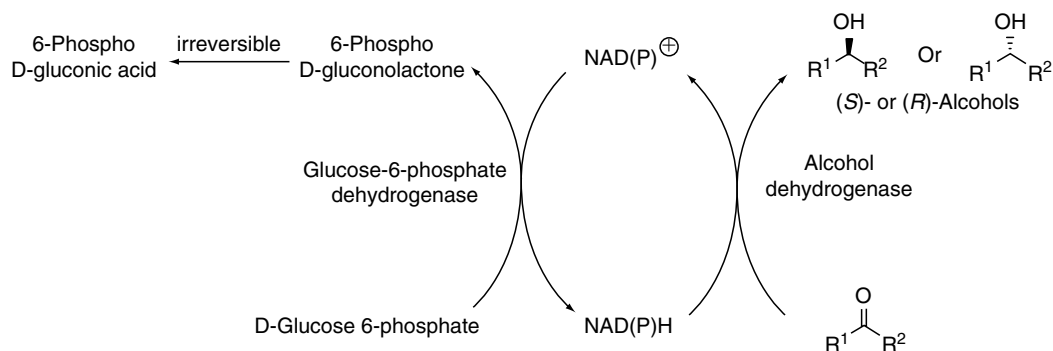
SCHEME 32.32

32.6 PROCESSES BASED ON COFACTOR REGENERATION WITH GLUCOSE-6-PHOSPHATE DEHYDROGENASE

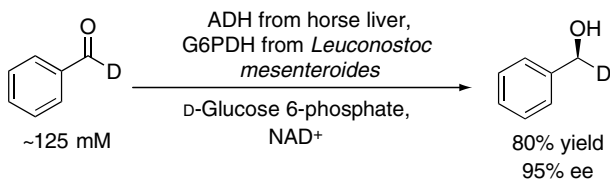
32.6.1 THE CONCEPT

Another concept for cofactor regeneration related to the one with a GDH is based on the use of a G6PDH. The synthesis of chiral alcohols by means of this methodology has already been reported in 1981 by Wong and Whitesides [116]. These types of enzymes can also be used for the regeneration of cofactors NADH and NADPH. The concept based on the use of such a G6PDH in combination with an ADH for the ketone reduction for the synthesis of optically active alcohols is shown in Scheme 32.33.

The high specific activity of G6PDHs, which is, for example, 676 U/mg for G6PDH from *Saccharomyces carlsbergensis*, contributes to their attractiveness as enzymes for cofactor regeneration. As a substrate, however, glucose-6-phosphate is needed, which can be formed by the cells starting from glucose (in case of a whole-cell approach) or used directly (in case of isolated enzymes). The latter option, however, is less attractive as glucose-6-phosphate



SCHEME 32.33



SCHEME 32.34

itself is an expensive compound. Accordingly, syntheses based on this type of cofactor regeneration with isolated enzymes have only been reported for small-scale applications. In contrast, the whole-cell approach has certainly the potential for large-scale applications, and promising process development has already been reported by Hanson et al. [117], which will be described in more detail in Section 32.6.2. An interesting option of this concept is the possibility to use intact cells, as glucose is transported to *E. coli* through a phosphotransferase system, which uses phosphoenol pyruvate as a phosphoryl donor, thus converting glucose into glucose-6-phosphate. Accordingly, a permeabilization of *E. coli* cells to ensure glucose supply (as in the case of the GDH-based cofactor regeneration) is not required.

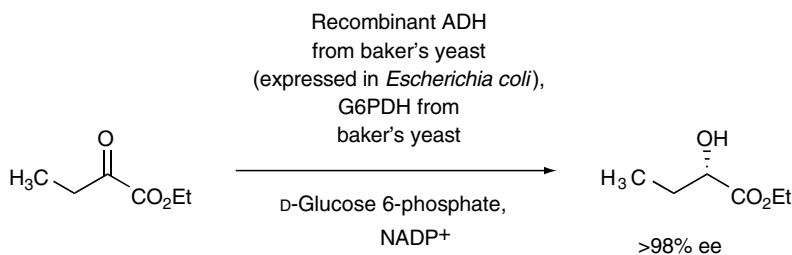
32.6.2 ISOLATED ENZYMES

The initial studies on this G6PDH-based cofactor regeneration system and its proof of principle for synthetic application have been carried out by the Whitesides group using isolated enzymes (Scheme 32.34) [116]. In this pioneering work, G6PDH from *Leuconostoc mesenteroides* in combination with added glucose-6-phosphate turned out to be highly suitable for cofactor regeneration of both cofactors, NAD^+ and NADP^+ . This G6PDH is stable and can be easily immobilized. The synthetic utility has already been shown in several examples, such as in the synthesis of (*S*)-1-*d*₁-phenylethanol which has been obtained in 80% yield with an ee of 95% at a substrate input of ~125 mM.

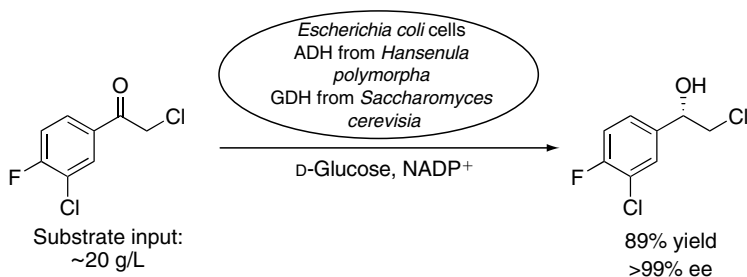
The combination of the G6PDH with the ADH from *L. kefir* for the synthesis of optically active (*R*)-phenylethan-1-ol has also been reported by Hummel [118]. The reductions, which were carried out at a 10 mM scale, gave the corresponding alcohol in 86% conversion.

The application of a two-phase solvent system for the reduction using an ADH and G6PDH has been described by Wong et al. for the synthesis of a variety of alcohols using an ADH from horse liver, *T. Brockii*, and a hydroxyl steroid ADH [119]. In the presence of an ADH from horse liver, ethyl and butyl esters of 4-keto hexanoate were reduced in an aqueous buffer–hexane two-phase system to the corresponding (*S*)-alcohol in quantitative yield and >98% ee.

A range of G6PDH-based reductions have been reported by the Stewart group, who applied their impressive set of 19 recombinant ADHs from *S. cerevisia* in a screening of 11 α - and β -keto ester substrates [120]. Notably, in all cases ADHs have been identified which are complementary to each other, thus delivering both types of enantiomers of the corresponding alcohols in optically pure form. A selected example is shown in Scheme 32.35. The reactions, which have been carried out at a 5 mM substrate, have been done in combination with a commercial G6PDH for cofactor regeneration during the reduction process [117]. Very recently, the Stewart group extended the application range of their recombinant ADHs from *S. cerevisia* toward a highly stereoselective enzymatic reduction of an α -chloro- β -keto ester [121]. The corresponding alcohol represents a key intermediate of the α -hydroxy- β -amino acid moiety of (–)-bestatin.



SCHEME 32.35



SCHEME 32.36

32.6.3 WHOLE-CELL REDUCTIONS

For the asymmetric reductions with *E. coli* whole cells bearing a G6PDH from *S. cerevisiae*, a recombinant whole cell was constructed recently by Hanson et al. [117]. Besides this G6PDH, the recombinant *E. coli* cells contained an NADPH-dependent ADH from *Hansenula polymorpha*, which turned out to give an ee of >99% in the target reduction of 2,3'-dichloro-4'-fluoroacetophenone. Subsequently, in the presence of a low amount of added NADP⁺ whole-cell biotransformation was carried out leading to the desired product (*S*)-2-chloro-1-(3'-chloro-4'-fluorophenyl)-ethanol in 89% yield and >99% ee (Scheme 32.36). The substrate input of this reduction was ~20 g/L, and the intact *E. coli* cells were provided with glucose directly.

32.7 SUMMARY AND OUTLOOK

In summary, numerous methodologies for asymmetric biocatalytic reductions of ketones have been developed based on the use of isolated enzymes and whole-cell catalysts. With respect to the latter, in particular, recombinant whole-cell systems very high importance. Notably, these reduction methods are based on different types of concepts for an *in situ* cofactor recycling, comprising substrate-coupled cofactor regeneration with 2-propanol, and enzyme-coupled cofactor regenerations with an FDH, GDH, and G6PDH. Although different from a conceptual point of view, high efficiency in organic synthetic transformations of ketones to optically active alcohols has been demonstrated by means of all these methods. This is underlined by several applications on industrial scale that have been achieved. In addition to efficient cofactor regeneration, easy, cost-attractive, and large-scale accessibility of the enzymes are another key criterion. Thus, availability of recombinant ADHs in

combination with the production of the biocatalysts by means of a high cell density fermentation process plays a crucial role. In the future, we will certainly see further screening for new ADHs and the extension of the application range of biocatalytic reduction.

REFERENCES AND NOTES

1. Kleemann, A., Engels, J., Kutscher, B., and Reichert, D., *Pharmaceutical Substances: Synthesis, Patents, Applications*, 4th ed., Thieme, Stuttgart, Germany, 2001.
2. Breuer, M., Ditrich, K., Harbicher, T., Hauer, B., Keßeler, M., Stürmer, M., and Zelinski, T., *Angew. Chem.*, 116, 804–843, 2004.
3. Helmchen, G., Hoffmann, R.W., Mulzer, J., and Schaumann, E., Eds., *Houben-Weyl, Methods of Organic Chemistry, Stereoselective Synthesis*, vol. E21, Thieme, Stuttgart, Germany, 1995.
4. Ojima, I., Ed., *Catalytic Asymmetric Synthesis*, 2nd ed., Wiley-VCH, Weinheim, Germany, 2000.
5. Jacobsen, E.N., Pfaltz, A., and Yamamoto, H., Eds., *Comprehensive Asymmetric Catalysis I–III*, Springer, Berlin, 1999.
6. Noyori, R., *Angew. Chem. Int. Ed.*, 41, 2008–2022, 2002.
7. Corey, E.J. and Helal, C.J., *Angew. Chem. Int. Ed.*, 37, 1986–2012, 1998.
8. Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M., and Witholt, B., *Nature*, 409, 258, 2001.
9. Nakamura, K. and Matsuda, T., in *Enzyme Catalysis in Organic Synthesis*, Drauz, K. and Waldmann, H., Eds., vol. 3, 2nd ed., Wiley-VCH, Weinheim, Germany, 2002, p. 991.
10. Hummel, W., *Adv. Biochem. Eng. Biotechnol.*, 58, 146, 1997.
11. Faber, K., *Biotransformations in Organic Chemistry*, vol. 5, Auflage, Springer, Berlin, 2004, Kapitel 2.2.3, p. 192.
12. Hummel, W., *TIBTECH*, 17, 487–492, 1999.
13. Eckstein, M., Daußmann, T., and Kragl, U., *Biocat. Biotransf.*, 22, 89, 2004.
14. Kula, M.-R. and Kragl, U., *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, p. 839.
15. Csuk, R. and Glanzer, B.I., *Chem. Rev.*, 91, 49, 1991.
16. Stewart, J.D., *Curr. Op. Drug Dis. Dev.*, 1, 278, 1998.
17. Csuk, R. and Glanzer, B.I., *Stereoselective Biocatalysis*, Patel, R.N., Ed., Marcel Dekker, New York, 2000, p. 527.
18. Stewart, J.D., *Curr. Op. Biotechnol.*, 1, 363, 2000.
19. Bertau, M. and Burli, M., Industrial applications of Baker's yeast reduction, *Chimia*, 54, 503, 2000.
20. Haberland, J., Hummel, W., and Dausmann, T., *Org. Proc. Res. Dev.*, 6, 458–462, 2002.
21. Haltrich, D., Nidetzky, B., Miemietz, G., Gollhofer, D., Lutz, S., Stolz, P., and Kulbe, K.D., *Biocat. Biotrans.*, 14, 31, 1996.
22. Holt, R.A., *Chimica Oggi*, 9, 17, 1996.
23. Holt, R.A. and Rigby, S.R., Avecia Limited, EP658211, 1995.
24. Liese, A., Seelbach, K., and Wandrey, C., *Industrial Biotransformations*, Wiley-VCH, Weinheim, Germany, 2000, p. 99.
25. Mertens, R., Greiner, L., van den Ban, E.C.D., Haaker, H.B.C.M., and Liese, A., *J. Mol. Catal. B Enzym.*, 24–25, 39, 2003.
26. Vrtis, J.M., White, A.K., Metcalf, W.M., and van der Donk, W.A., *Angew. Chem.*, 114, 3391, 2002.
27. Naahmniek, S., Hummel, W., and Gröger, H., Degussa AG, DE10240603, 2004.
28. Yuan, R., Watanabe, S., Kuwabata, S., and Yoneyama, H., *J. Org. Chem.*, 62, 2494, 1997.
29. Homann, M.J., Vail, R.B., Previte, E., Tamarez, M., Morgan, B., Dodds, D.R., and Zaks, A., *Tetrahedron*, 60, 789–797, 2004.
30. Villela Filho, M., Stillger, T., Müller, M., Liese, A., and Wandrey, C., *Angew. Chem. Int. Ed.*, 42, 2993–2996, 2003.
31. Stillger, T., Bönitz, M., Vilella, M., and Liese, A., *Chemie Ingenieur Technik*, 74, 1035, 2002.

32. Stampfer, W., Kosjek, B., Moitzi, C., Kroutil, W., and Faber, K., *Angew. Chem. Int. Ed.*, 41, 1014, 2002.
33. Nakamura, K., Inoue, Y., Matsuda, T., and Misawa, I., *J. Chem. Soc. Perkin Trans. 1*, 2397, 1999.
34. Bradshaw, C.W., Fu, H., Shen, G.-J., and Wong, C.-H., *J. Org. Chem.*, 57, 1526, 1992.
35. Yang, H., Jönsson, A., Wehtje, E., Adlerkreutz, P., and Mattiasson, B., *Biochim. Biophys. Acta*, 1336, 51, 1997.
36. Clair, N. St, Wang, Y.-F., and Margolin, A.L., *Angew. Chem. Int. Ed.*, 39, 380, 2000.
37. Gupta, A., Breese, K., Bange, G., and Neubauer, P., Juelich Enzyme Products GmbH, WO 02086126, 2002.
38. De Temino, D.M.-R., Hartmeier, W., and Ansorge-Schumacher, M.B., *Enzyme Microb. Technol.*, 36, 3, 2005.
39. Riermeier, T., Bornscheuer, U., Altenbuchner, J., and Hildebrandt, P., Degussa AG, EP 1241263, 2002.
40. Hildebrandt, P., Riermeier, T., Altenbuchner, J., and Bornscheuer, U., *Tetrahedron Asymmetry*, 12, 1207, 2001.
41. Inoue, K., Makino, Y., and Itoh, N., *Appl. Environ. Microbiol.*, 71, 3633, 2005.
42. Inoue, K., Makino, Y., and Itoh, N., *Tetrahedron Asymmetry*, 16, 2539, 2005.
43. Eckstein, M., Vilella, M., Liese, A., and Kragl, U., *Chem. Commun.*, 1984, 2004.
44. Matsumura, S., Imafuku, H., Takahashi, Y., and Toshima, K., *Chem. Lett.*, 251, 1993.
45. Itoh, N., Matsuda, M., Mabuchi, M., Dairi, T., and Wang, J., *Eur. J. Biochem.*, 269, 2394, 2002.
46. Matsuyama, A., Yamamoto, H., and Kobayashi, Y., *Org. Process Res. Dev.*, 6, 558, 2002.
47. Amidjojo, M. and Weuster-Botz, D., *Tetrahedron Asymmetry*, 16, 899, 2005.
48. Wolberg, M., Hummel, W., Wandrey, C., and Müller, M., *Angew. Chem.*, 112, 4476, 2000.
49. Stampfer, W., Kosjek, B., Faber, K., and Kroutil, W., *J. Org. Chem.*, 68, 402, 2003.
50. Stampfer, W., Kosjek, B., Kroutil, W., and Faber, K., *Biotechnol. Bioeng.*, 81, 865, 2003.
51. Stampfer, W., Kosjek, B., Kroutil, W., Faber, K., Niehaus, F., and Eck, J., Ciba Specialty Chemicals Holding Inc., WO2005026338, 2005.
52. Slusarczyk, H., Felber, S., Kula, M.-R., and Pohl, M., *Eur. J. Biochem.*, 267, 1280, 2000.
53. Kroner, K.H., Schütte, H., Stach, W., and Kula, M.R., *J. Chem. Technol. Biotechnol.*, 32, 130, 1982.
54. Wandrey, C., Wichmann, R., Bückmann, A.F., and Kula, M.-R., *Enzyme Eng.*, 5, 453, 1980.
55. Shaked, Z. and Whitesides, G.M., *J. Am. Chem. Soc.*, 102, 7104, 1980.
56. Bommarius, A.S., Drauz, K., Hummel, W., Kula, M.-R., and Wandrey, C., *Biocatalysis*, 10, 37, 1994.
57. Liese, A., Seelbach, K., and Wandrey, C., *Industrial Biotransformations*, Wiley-VCH, Weinheim, Germany, 2000, p. 103.
58. Tishkov, V.I., Galkin, A.G., Fedorchuk, V.V., Savitsky, P.A., Rojkova, A.M., Gieren, H., and Kula, M.-R., *Biotechnol. Bioeng.*, 64, 187, 1999.
59. Galkin, A., Kulakova, L., Tishkov, V., Esaki, N., and Soda, K., *Appl. Microbiol. Biotechnol.*, 44, 479, 1995.
60. Ernst, M., Kaup, B., Müller, M., Binger-Meyer, S., and Sahm, H., *Appl. Microbiol. Biotechnol.*, 66, 629, 2005.
61. Hummel, W. and Gottwald, C., Bayer AG, DE4209022, 1992.
62. Zelinski, T., Peters, J., and Kula, M.-R., *J. Biotechnol.*, 33, 283, 1994.
63. Peters, J., Minuth, T., and Kula, M.-R., *Enzyme Microb. Technol.*, 15, 950, 1993.
64. Adlerkreutz, P., *Enzyme Microb. Technol.*, 13, 9, 1991.
65. Itoh, N., Mizuguchi, N., and Mabuchi, M., *J. Mol. Catal. B Enzym.*, 6, 41, 1999.
66. Peters, J., Zelinski, T., Minuth, T., and Kula, M.-R., *Tetrahedron Asymmetry*, 4, 1683, 1993.
67. Peters, J., Zelinski, T., and Kula, M.-R., *Appl. Microbiol. Biotechnol.*, 38, 334, 1992.
68. Zelinski, T., Liese, A., Wandrey, C., and Kula, M.-R., *Tetrahedron Asymmetry*, 10, 1681, 1999.
69. Kruse, W., Hummel, W., and Kragl, U., *Recl. Trav. Chim. Pays-Bas*, 115, 239, 1996.
70. Kragl, U., Kruse, W., Hummel, W., and Wandrey, C., *Biotechnol. Bioeng.*, 52, 309, 1996.
71. Liese, A., Seelbach, K., and Wandrey, C., *Industrial Biotransformations*, Wiley-VCH, Weinheim, Germany, 2000, p. 103.

72. Kruse, W., Kragl, U., and Wandrey, C., Forschungszentrum Jülich GmbH, DE 4436149, 1996.
73. Liese, A., Zelinski, T., Kula, M.-R., Kierkels, H., Karutz, M., Kragl, U., and Wandrey, C., *J. Mol. Catal. B Enzym.*, 4, 91, 1998.
74. Rissom, S., Beliczey, J., Giffels, G., Kragl, U., and Wandrey, C., *Tetrahedron Asymmetry*, 10, 923, 1999.
75. Gröger, H., Hummel, W., Buchholz, S., Drauz, K., Nguyen, T.V., Rollmann, C., Hüskens, H., and Abokitse, K., *Org. Lett.*, 5, 173, 2003.
76. Gröger, H., Hummel, W., Rollmann, C., Chamouleau, F., Hüskens, H., Werner, H., Wunderlich, C., Abokitse, K., Drauz, K., and Buchholz, S., *Tetrahedron*, 60, 633, 2004.
77. Gröger, H., Rollmann, C., Hüskens, H., Werner, H., Chamouleau, F., Hagedorn, C., Drauz, K., and Hummel, W., Degussa AG, WO 2004085662, 2004.
78. Gröger, H., *Chiral Europe Conference Proceedings*, 2004.
79. Nanduri, V.B., Banerjee, A., Howell, J.M., Brzozowski, B.B., Eiring, R.F., and Patel, R.N., *J. Ind. Microbiol. Biotechnol.*, 25, 171, 2000.
80. Orlich, B. and Schomäcker, R., *Biotechnol. Bioeng.*, 65, 357, 1999.
81. Orlich, B., Berger, H., Lade, M., and Schomäcker, R., *Biotechnol. Bioeng.*, 70, 638, 2000.
82. Matsuyama, A., Yamamoto, H., and Kobayashi, Y., *Org. Proc. Res. Dev.*, 6, 558, 2002.
83. Ernst, M., Kaup, B., Bringer-Meyer, S., and Sahm, H., *Appl. Microbiol. Biotechnol.*, 66, 629, 2005.
84. Weckbecker, A. and Hummel, W., *Biotechnol. Lett.*, 26, 1739, 2004.
85. Hummel, W. and Kula, M.-R., Forschungszentrum Jülich GmbH, EP 456107, 1991.
86. Bradshaw, C.W., Hummel, W., and Wong, C.-H., *J. Org. Chem.*, 57, 1532, 1992.
87. Haltrich, D., Nidetzky, B., Miemitz, D., Gollhofer, D., Lutz, S., Stolz, P., and Kulbe, K.D., *Biocatal. Biotrans.*, 14, 31, 1996.
88. Kataoka, M., Rohani, L.P.S., Wada, M., Kita, K., Yanase, H., Urabe, I., and Shimizu, S., *Biosci. Biotechnol. Biochem.*, 62, 167, 1998.
89. Hilt, W., Pfeleiderer, G., and Fortnagel, P., *Biochim. Biophys. Acta*, 1076, 298, 1991.
90. Karmali, A. and Serralheiro, L., *Biochimie*, 70, 1401, 1988.
91. Bach, J.A. and Sadoff, H.L., *J. Bacteriol.*, 83, 699, 1962.
92. Wong, C.-H., Drueckhammer, D.G., and Sweers, H.M., *J. Am. Chem. Soc.*, 107, 4028, 1985.
93. Smith, L.D., Budgen, N., Bungard, S.J., Danson, M.J., and Jough, D.W., *Biochem. J.*, 261, 973, 1989.
94. Wong, C.-H., Drueckhammer, D.G., and Sweers, H.M., *J. Am. Chem. Soc.*, 107, 4028, 1985.
95. Wong, C.-H. and Drueckhammer, D.G., *Biol. Technol.*, 3, 649, 1995.
96. Bastos, F.M., dos Santos, A.G., Jones J. Jr., Oestreicher, E.G., Pinto, G.F., and Paiva, L.M.C., *Biotechnol. Technol.*, 13, 661, 1999.
97. Zhu, D., Mukherjee, C., and Hua, L., *Tetrahedron Asymmetry*, 16, 3275, 2005.
98. Patel, R.N., Banerjee, A., McNamee, C.G., Brzozowski, D., Hanson, R.L., and Szarka, L.J., *Enzyme Microb. Technol.*, 15, 1014, 1993.
99. Liese, A., Seelbach, K., and Wandrey, C., *Industrial Biotransformations*, Wiley-VCH, Weinheim, Germany, 2000, p.107.
100. Kaluzna, I.A., Rozzell, J.D., and Kambourakis, S., *Tetrahedron Asymmetry*, 16, 3682, 2005.
101. Kataoka, M., Kita, K., Wada, M., Yasohara, Y., Hasegawa, J., and Shimizu, S., *Appl. Microbiol. Biotechnol.*, 62, 437, 2003.
102. Yamada, H., Shimizu, S., Kataoka, M., Sakai, H., and Miyoshi, T., *FEMS Microbiol. Lett.*, 70, 45, 1990.
103. Kataoka, M., Sakai, H., Morikawa, T., Katoh, M., Miyoshi, T., Shimizu, S., and Yamada, H., *Biochim. Biophys. Acta*, 1122, 57, 1992.
104. Kita, K., Matuszaki, K., Hashimoto, T., Yanase, H., Kato, N., Chung, M.C.M., Kataoka, M., and Shimizu, S., *Appl. Environ. Microbiol.*, 62, 2303, 1996.
105. Wada, M., Kataoka, M., Kawabata, H., Yasohara, Y., Kizaki, N., Hasegawa, J., and Shimizu, S., *Biosci. Biotechnol. Biochem.*, 62, 280, 1998.
106. Yasohara, Y., Kizaki, N., Hasegawa, J., Wada, M., Kataoka, M., and Shimizu, S., *Biosci. Biotechnol. Biochem.*, 64, 1430, 2000.
107. Shimizu, S., Kataoka, M., Katoh, M., Morikawa, T., Miyoshi, T., and Yamada, H., *Appl. Environ. Microbiol.*, 56, 2374, 1990.

108. Kataoka, M., Rohani, L.P.S., Yamamoto, K., Wada, M., Kawabata, H., Kita, K., Yanase, H., and Shimizu, S., *Appl. Microbiol. Biotechnol.*, 48, 699, 1997.
109. Kataoka, M., Rohani, L.P.S., Wada, M., Kita, K., Yanase, H., Urabe, I., and Shimizu, S., *Biosci. Biotechnol. Biochem.*, 62, 167, 1998.
110. Kataoka, M., Yamamoto, K., Kawabata, H., Wada, M., Kita, K., Yanase, H., and Shimizu, S., *Appl. Microbiol. Biotechnol.*, 51, 486, 1999.
111. Kizaki, N., Yasohara, Y., Hasegawa, J., Wada, M., Kataoka, M., and Shimizu, S., *Appl. Microbiol. Biotechnol.*, 55, 590, 2001.
112. Yasohara, Y., Kizaki, N., Hasegawa, J., Wada, M., Kataoka, M., and Shimizu, S., *Tetrahedron Asymmetry*, 12, 1713, 2001.
113. Patel, R.N., Goswami, A., Chu, L., Donovan, M.J., Nanduri, V., Goldberg, S., Johnston, R., Siva, P.J., Nielsen, B., Fan, J., He, W., Shi, Z., Wang, K.Y., Eiring, R., Cazzulino, D., Singh, A., Mueller, R., *Tetrahedron Asymmetry*, 15, 1247–1258, 2004.
114. Weckbecker, A. and Hummel, W., *Methods in Biotechnology, Microbial Enzymes and Biotransformations*, Barredo, J.L., Ed., vol. 17, Humana Press, Totowa, NJ, 2004, p. 225.
115. Gröger, H., Chamouleau, F., Orologas, N., Rollmann, C., Drauz, K., Hummel, W., Weckbecker, A., and May, O., *Angew. Chem.*, 2006, in press.
116. Wong, C.-H. and Whitesides, G.M., *J. Am. Chem. Soc.*, 103, 4890, 1981.
117. Hanson, R.L., Goldberg, S., Goswami, A., Tully, T.P., and Patel, R.N., *Adv. Synth. Catal.*, 347, 1073, 2005.
118. Hummel, W., *Appl. Microbiol. Biotechnol.*, 34, 15, 1990.
119. Drueckhammer, D.J., Sadozai, S.K., Wong, C.-H., and Roberts, S.M., *Enzyme Microb. Technol.*, 9, 564–570, 1987.
120. Kaluzna, I.A., Matsuda, T., Sewell, A.K., and Stewart, J.D., *J. Am. Chem. Soc.*, 126, 12827–12832, 2004.
121. Feske, B.D. and Stewart, J.D., *Tetrahedron Asymmetry*, 16, 3124–3127, 2005.

33 Enzyme Catalysis in Nonaqueous Media: Past, Present, and Future

*Susanne Dreyer, Julia Lembrecht, Jan Schumacher,
and Udo Kragl*

CONTENTS

33.1	Introduction	791
33.2	EC 1: Oxidoreductases	793
33.2.1	Alcohol Dehydrogenases	794
33.2.2	Laccases	797
33.2.3	Monooxygenases	798
33.3	EC 2: Transferases	800
33.4	EC 3: Hydrolases	804
33.4.1	Immobilization	807
33.4.2	Ionic Liquids	807
33.4.3	Reverse Micelles	808
33.4.4	Supercritical Fluids	809
33.4.5	Other Hydrolytic Enzymes	811
33.5	EC 4: Lyases	812
33.5.1	Nitrile Hydratases	813
33.5.2	Hydroxynitrile Lyases	814
33.5.3	Aldolases	816
33.6	EC 5: Isomerases	817
33.7	EC 6: Ligases	819
	References	820

33.1 INTRODUCTION

Nature has designed its biocatalysts to perform best in an aqueous surrounding, neutral pH, and temperatures below 50°C. However, these conditions are often contrary to the requirements of the process engineer or chemist to optimize a reaction with respect to volumetric productivity or an easy downstream processing when substrates and/or products are not easily soluble in water. To overcome these bottlenecks addition of organic solvents is common practice. This has been used by various authors over the years, but a breakthrough was achieved during the second half of the 20th century [1–3].

The solvent can either be water-miscible or water-immiscible, resulting in one- or two-phase systems. Depending on the solvent phase the enzyme will be dissolved or only suspended. Thus, the systems might be liquid, liquid–liquid, or liquid–solid, but even gas-phase reactions are gaining increasing attention. The possibilities are summarized in Figure 33.1. The aim of this chapter is to highlight some of the developments over the years. Thus, in no way can it be exhaustive.

In a pure aqueous system the solvents implied are the dissolved enzyme(s), cofactors and cosubstrates if needed, the substrate, and the product. In a pure organic phase most often the enzyme is not soluble and therefore applied in suspension either in its native form or on a carrier. In a two-phase system formed by water and an organic solvent the enzyme is either dissolved in the bulk aqueous phase or can be localized at the interface. The organic phase then can be seen as a reservoir for a poorly water-soluble substrate. During the reaction the substrate and the product formed will partition between the two phases. On the one hand, using two-phase systems for enzyme-catalyzed reactions might have several advantages. Firstly, higher concentrations of hydrophobic substrates and therefore higher volumetric productivities can be achieved. Secondly, no enzyme inhibition by high substrate concentrations will occur. Thirdly, an easy workup by phase separation is possible. Fourthly, “free immobilization” of the enzyme and cofactor in the aqueous phase allows reuse of the enzyme and the cofactor [4–6].

On the other hand, the influence of the organic phase on enzyme activity, stability, and regio- and stereospecificity has to be evaluated. The choice of the organic solvent is mainly influenced by the enzyme behavior and by the partitioning behavior of substrate and product [7–9]. It should be noted that despite all success there is no general rule as to which solvent is “enzyme friendly.” To a certain extent, the log *P* concept, based on the distribution

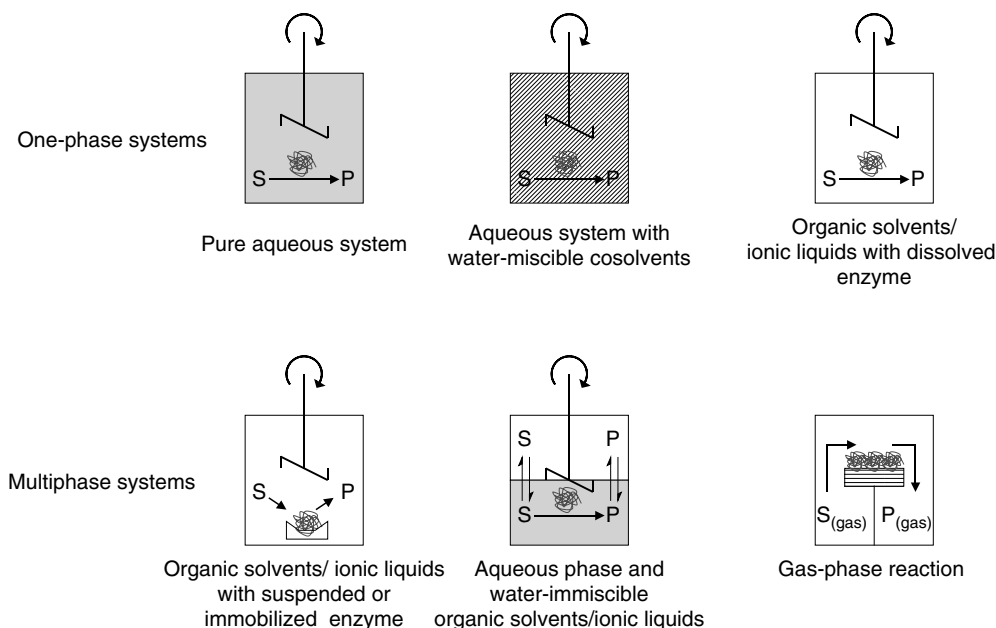


FIGURE 33.1 Possible reaction system for biocatalysis in nonconventional media.

coefficient between water and *n*-octanol, can be used as a guideline [10]. In general, solvents with a log *P* > 3 such as xylene (3.1) or hexane (3.9) are less deactivating than those with a low log *P* such as ethanol (−0.24). Surprisingly, *tert*-butanol (0.35) stabilizes enzymes [11]. Certainly the hydrophilicity of the cosolvent is important, as it allows interaction and breaking of hydrogen bonds that are stabilizing the tertiary structure of the enzyme. However, not only have common organic solvents been used for biocatalysis, but supercritical CO₂ [12] and recently even ionic liquids (ILs) have also been shown to be compatible with enzymes or whole cells [13–16].

33.2 EC 1: OXIDOREDUCTASES

The oxidoreductases represent the first group of the classification formulated by the enzyme community (EC numbers). Enzymes that are part of this group reversely catalyze the oxidation and reduction of substrates by transferring two electrons. Mostly they need a cofactor, also called coenzyme, which activates the enzyme and acts as a mediator between the active site of the biocatalyst and the substrate [17]. These cofactors are small organic nonprotein molecules that can covalently or noncovalently bind to the inactive apoenzyme to build the active catalyst (haloenzyme). Such coenzymes are molecules like nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), and flavine adenine dinucleotide (FAD). As can be seen in Table 33.1, oxidoreductases are divided into 22 subclasses.

TABLE 33.1
Subclasses of the Oxidoreductases

EC 1 Oxidoreductases	
EC 1.1	Acting on the CH–OH group of donors
EC 1.2	Acting on the aldehyde or oxo group of donors
EC 1.3	Acting on the CH–CH group of donors
EC 1.4	Acting on the CH–NH ₂ group of donors
EC 1.5	Acting on the CH–NH group of donors
EC 1.6	Acting on the NADH or NADPH
EC 1.7	Acting on the other nitrogenous compounds of donors
EC 1.8	Acting on a sulfur group of donors
EC 1.9	Acting on a heme group of donors
EC 1.10	Acting on diphenols and related substances of donors
EC 1.11	Acting on peroxide as acceptor
EC 1.12	Acting on hydrogen of donors
EC 1.13	Acting on single donors with incorporation of molecular oxygen (oxygenases)
EC 1.14	Acting on paired donors with incorporation or reduction of molecular oxygen
EC 1.15	Acting on superoxide as acceptor
EC 1.16	Oxidizing metal ions
EC 1.17	Acting on the CH or CH ₂ groups
EC 1.18	Acting on iron–sulfur proteins as donor
EC 1.19	Acting on reduced flavodoxin as donor
EC 1.20	Acting on phosphorus or arsenic in donor
EC 1.21	Acting on X–H and Y–H to form an X–Y bond
EC 1.97	Other oxidoreductases

33.2.1 ALCOHOL DEHYDROGENASES

The biggest subclass of the oxidoreductases comprise alcohol dehydrogenases (ADHs; EC 1.1). In the past, the ADHs were typically used in a buffered aqueous system because they and their cofactors are sensitive against organic solvents. For example, Hummel published the reduction of acetophenone to (*R*)-1-phenylethanol by the ADH from *Lactobacillus kefir* in potassium phosphate buffer with NADPH regenerated by a glucose-6-phosphate dehydrogenase (G6PDH) [18].

An oft-applied enzyme is the yeast alcohol dehydrogenase (YADH). The development started with the use of whole cells as biocatalysts to avoid the cofactor regeneration in an additional reaction step. Since the end of the 20th century YADH has also been deployed in nonconventional media. Howarth et al. reported that it is possible to use immobilized baker's yeast as whole cells in ILs mixed with water (10:1) to reduce prochiral ketones [19,20]. Organic solvents offer the chance to selectively form one of the enantiomers. It has been reported that both enantiomers were produced when reducing 2-oxohexanolate by baker's yeast in water, but when the biotransformation was conducted in benzene, the (*R*)-alcohol was formed in high yields [21]. Although the ADH can be successfully used under nonconventional conditions, it should be noted that the catalytic activity strongly depends on the water activity in the system [22].

Further, Liao et al. performed a YADH covalently bonded onto Fe₃O₄ magnetic nanoparticles, which is active in a water/AOT/isooctane microemulsion (Figure 33.2). The residual activity immobilized YADH after 700 h was 78% and, in contrast to that, the free YADH loses most of its activity in the same reaction system after 1 h [23]. Beyond this, YADH is highly stable and active in reverse micelles of AOT/isooctane depending on water content, pH, and time [24,25].

For further research cross-linked enzyme aggregates (CLEAs), gas-phase reactions, and combinations of different organic solvents would be a promising field. However, there are still some open questions in the areas of one- and two-phase systems that have to be analyzed for a better understanding of the mechanism.

Since the last 10 to 15 years there have appeared a number of reports about ADHs in nonconventional media. A new ADH from *L. brevis* (LBADH), first mentioned by Riebel,

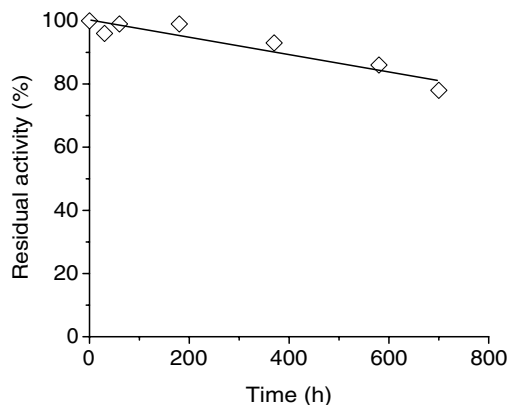


FIGURE 33.2 Storage stability of bound YADH at 25°C. The activity measurement was preformed in 10 ml microemulsion solution at 0.1 M AOT, 0.2 mM NADH, 0.1 M 2-butanone, 25°C, and $\omega_0 = 25$. The concentration of bound YADH was 0.5 mg/ml. The initial 100% absolute values of activity for bound YADH was 8 nmol/(min·mg). (From Liao, M.H. and Chen, D.H., *J. Mol. Catal. B Enzym.*, 18, 81–87, 2002.)

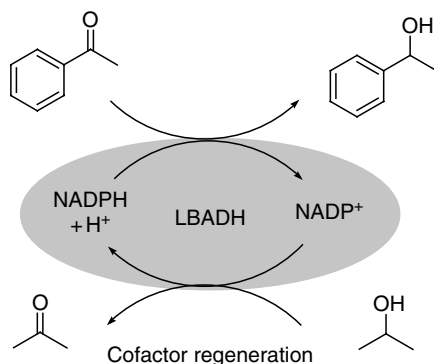


FIGURE 33.3 Model reaction in the gas phase: reduction of acetophenone with cofactor regeneration by 2-propanol oxidation. Enzyme and cofactor are immobilized on glass beads. (From Ferloni, C. et al., *Biokatalyse* (Transkript Sonderheft), 105–108, 2003.)

is able to catalyze the reduction of prochiral ketones [26]. This can be done not only in buffer but also in many different reaction systems. The advantage of all these systems is a higher stability of the LBADH. As published by Filho et al. the half-lives of ADHs are not directly related to the log *P* values of the solvent, but it is possible to appraise the miscibility with water and thus enable the contact between the enzyme and the organic solvent [27].

Ferloni et al. reported that the immobilized LBADH shows an enzyme activity of 100% for the reduction of substrates in the aqueous phase and also in the gas phase (Figure 33.3) [28]. This implies a high reactivity of the ADH to the reduction of acetophenone in the gas phase. The conversion of the substrate and the reactivity of the enzyme depend on relative moisture in the reaction system, on the pressure, and on the molecular ratio of the cosubstrate 2-propanol to the substrate.

The reduction of prochiral ketons catalyzed by LBADH including substrate-coupled regeneration with 2-propanol has been used for the production of chiral alcohol on a 10 to 100 kg scale, e.g., ethyl-(*R*)-3-hydroxybutyrate or (*R*)-2-octanol. The reaction was performed in a one-phase system with an extraction step at the end [29]. Schumacher et al. researched the LBADH-catalyzed reduction of short aliphatic ketones in a one-phase system with cosolvents [30]. They investigated an increasing enantiomeric excess (ee) of (*R*)-butan-2-ol (36.5 to 43.0%) by increasing the amount of acetonitrile from 0% (v/v) to 24.5% (v/v).

It is also possible to arrange the reaction in a two-phase system (Figure 33.4). Eckstein et al. investigated the reduction of 2-octanone to (*R*)-2-octanol with the cofactor regeneration by the same enzyme in two different binary systems [31]. In the first system of methyl *tert*-butyl ether (MTBE) and buffer the reduction catalyzed by the free LBADH achieved a conversion of 61% after 180 min. In contrast, the reaction is much faster when using an IL instead of an organic solvent as second phase. In the case of [BMIM][(CF₃SO₂)₂N]/buffer the conversion after 180 min reaches 88%.

Using the LBADH in a two-phase system has two advantages. Firstly, the concentration of poorly water-soluble substrates can be raised in the total system for even decoupling as reported by Kroutil et al. [32]. Secondly, there is an increase of stability of the LBADH in comparison to conversion in one-phase systems with cosolvents. It depends on the organic solvent in comparison to the conversion in buffer [27]. The half-life of the LBADH is effected by the nature of the organic solvent that is used as second phase, although the contact between the enzyme and the organic media should be marginal (Figure 33.5) [33].

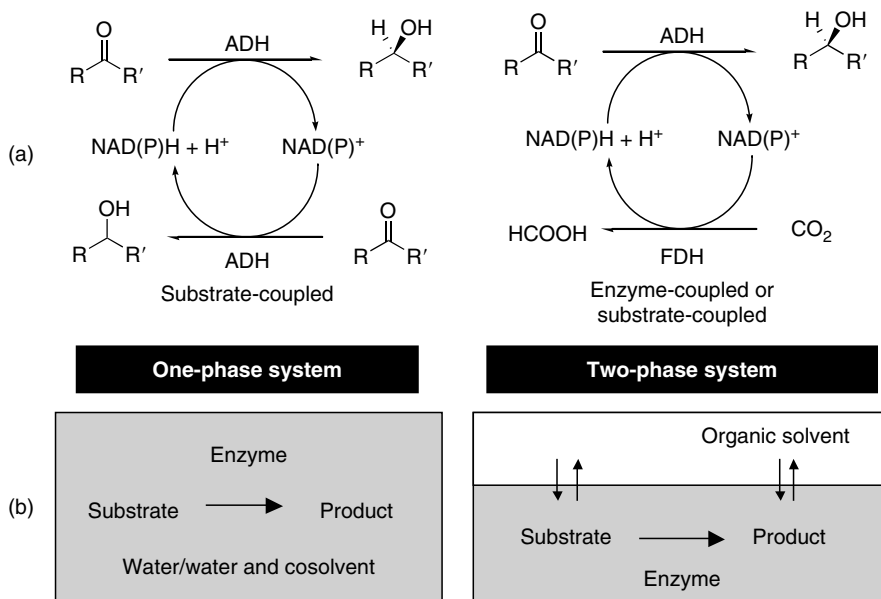


FIGURE 33.4 (a) Cofactor regeneration with substrate-coupled and enzyme-coupled approach. (b) Scheme of a one-phase system and a two-phase system for enzyme catalysis. (From Eckstein, M. et al., *Chem. Comm.*, 1084–1085, 2004).

The enzymatic reduction by LBADH requires NADPH, an expensive cofactor. To configure this reaction economically an *in situ* regeneration is necessary. Eckstein et al. reviewed various methods for one- and two-phase systems to minimize the required cofactor concentration [29]. A common method is the use of a second substrate, mostly 2-propanol, to recycle the cofactor into its catalytically active form. This substrate-coupled regeneration is

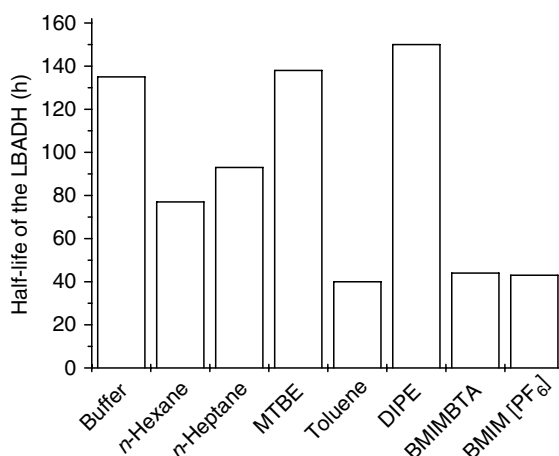


FIGURE 33.5 Half-life of the LBADH in buffer and different two-phase systems. Conditions: total volume 8 ml, organic solvent/water 1:1 ($\alpha = 1$), 3 U/ml (aq) LBADH, 0.1 mM (aq) $\text{NADP}^+/\text{NADPH}$, 30°C, 900 rpm, phosphate buffer (50 mM, 1 mM MgCl_2 , pH 7.0). (From Lembrecht, J., unpublished data, 2006.)

mostly implemented by the same enzyme as that for the reduction of the ketone. Alternatively, an enzyme-coupled regeneration can be used. In this case, a second enzyme is deployed to restore NAD(P)H. Firstly, a regeneration by formate dehydrogenase (FDH) that is able to oxidize formate to CO₂ [34] has been reported, but also an application of glucose dehydrogenase (GDH), which oxidizes D-glucose, or D-glucose-6-phosphate dehydrogenase, which converts D-glucose-6-phosphate, was mentioned in literature. These are the two mainly used regeneration methods for the reduction of prochiral ketones in one- and two-phase systems [35].

Another possibility to permit the production of chiral hydrophobic alcohols catalyzed by ADHs in nonconventional media is the immobilization on a support. De Temiño et al. reported a higher stability of ADH from *L. kefir* by entrapping the enzyme and its cofactor in polyvinyl alcohol gel beads [36]. In the case of an immobilized enzyme it is possible to convert in pure organic solvents. An encapsulation in reverse micelles also causes a higher half-life time of ADHs in pure organic solvents [25]. To create a higher stability of the LBADH the preparation of CLEAs has been mentioned by Mateo et al., although the cross-linked ADH exhibits a recovered activity of 7 to 10% relative to the native enzyme cross-linked with dextran polyaldehyde [37].

To facilitate the catalysis in pure organic solvents or in the environment-friendly ILs genetic rearrangement of the enzyme would be a possibility.

33.2.2 LACCASES

Laccases are the second group of oxidoreductases that will be discussed in this chapter. These copper-containing enzymes oxidize substrates by reduction of molecular oxygen to water. Typically, laccases are deployed in textile, pulp, and paper industries, but they can be used as “green” catalysts in organic synthesis as well. Khmelnitsky studied the degeneration of laccase from *Polyporus versicolor* and other enzymes for the oxidation of a model substrate in many different organic solvents [38]. Laccases, which are very sensitive to nonconventional conditions, are only active in organic solvents till a limiting concentration of the cosolvent is exceeded. This barrier depends on the enzyme and also on the cosolvent; so the border where the enzyme is still native (catalytically active) must be determined for each protein.

Commonly, laccases are used in buffer solution, e.g., in the oxidative polymerization of 4-chloroguaiacol [39]. It has been found that a pH between 4 and 5 is the optimized condition for the described reaction.

As a result of the inactivation by organic solvents immobilization is a typical method for the laccase-catalyzed synthesis in organic solvents. Pilz et al. published the synthesis of coupling products of phenolic substrates (Figure 33.6) in different reactors [40]. They used

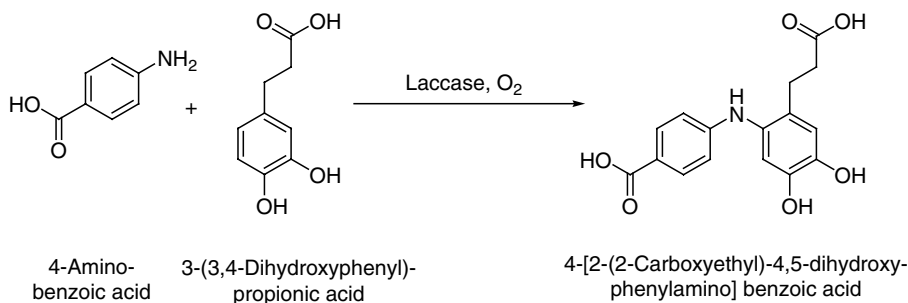


FIGURE 33.6 Cross-coupling reaction catalyzed by laccase from *Pycnoporus cinnabarinus*. (From Pilz, R. et al., *Appl. Microbiol. Biotechnol.*, 60, 708–712, 2003.)

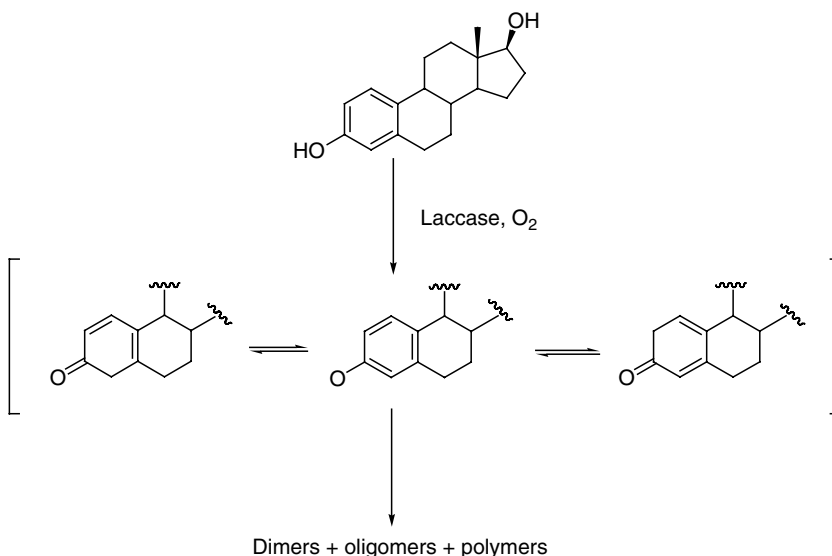


FIGURE 33.7 Laccase-mediated oxidation of β -estradiol. (From Nicotra, S. et al., *Tetrahedron Asymmetry*, 15, 2927–2931, 2004.)

an immobilized laccase prepared from the white rot fungus *Pycnoporus cinnabarinus* in a stirred tank reactor (STR) and also in a continuously operated enzyme-membrane reactor (EMR) in acetate buffer (pH 5). Immobilization of the enzyme on a support offers the possibility to reduce the amount of enzyme required for the reaction.

It has also been reported that the oxidation of the steroid hormone 17 β -estradiol by laccase from *Mycellyphthora* and *Trametes pubescens* in organic media is feasible. Nicotra et al. accomplished the laccase-mediated oxidation in pure organic solvents and also in two-phase systems using the enzyme immobilized on glass beads (Figure 33.7) [41]. As pure solvent a mixture of dioxin and water-saturated toluene has been applied and as two-phase system AcOEt/buffer (pH 4.5) was utilized. The oxidation of β -estradiol by laccase from *P. versicolor* was described more than 30 years ago in one of the first papers on the use of enzymes in two-phase systems [42].

In the future it might be possible to use laccases not only in the paper industry but also to produce new polymers that cannot be synthesized by organometallic catalysts or other enzymes. Therefore, a further development of immobilization methods to generate highly active and stable laccases in organic media is necessary. Perhaps it would be possible to deploy immobilized laccases in a gas-phase reaction.

33.2.3 MONOOXYGENASES

The third subclass of oxidoreductases that will be discussed is made up of monooxygenases. This subclass of the EC 1 catalyzes the oxidation of unsaturated substrates to oxiranes or lactones by reducing molecular oxygen. Monooxygenases are dependent on cofactors, mostly NADH and NADPH, so around 10 years ago the deployment of whole cells as biocatalyst was common. Schmid et al. availed whole cells containing styrene monooxygenase recombinant in *Escherichia coli*. [43]. The synthesis of (*S*)-styrenes from styrene and its derivatives was arranged in a two-phase system containing buffer and dioctyl phthalate. Because of the

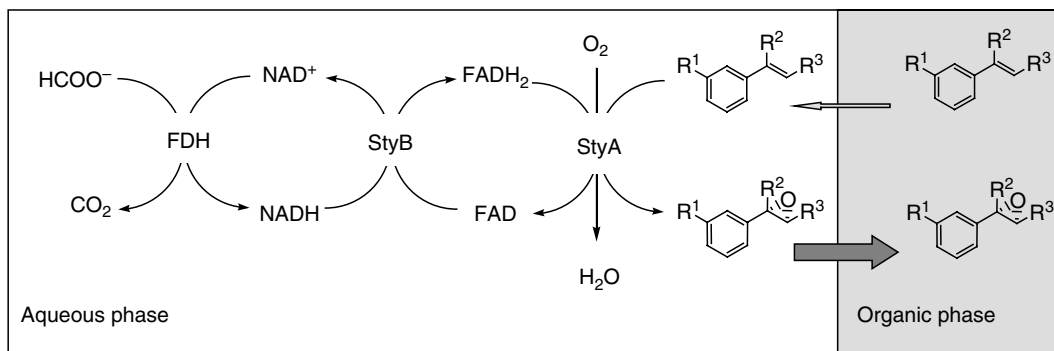


FIGURE 33.8 Reaction pathway during biocatalytic epoxidation in a two-phase system. The organic phase serves as a substrate reservoir and product sink. In the aqueous phase, formate dehydrogenase (FDH) and formate were used for regeneration of NADH. StyB transfers the reducing equivalents from NADH to flavine adenine dinucleotide (FAD). FADH₂ and oxygen are cosubstrates for olefin epoxidation by StyA. R¹ = H, Cl; R² = R³ = H, CH₃. (From Hofstetter, K. et al., *Angew. Chem. Int. Ed.*, 43, 2163–2166, 2004.)

toxicity of the substrates the second phase could work as a reservoir to keep the substrate concentration low in the environment of the enzyme. The two-phase system also allows an *in situ* product removal (IPS) to isolate the toxic and water-labile product.

A biocatalytic asymmetric epoxidation with NADH regeneration in organic–aqueous emulsions has also been published [44]. As can be seen in Figure 33.8 there are two styrene monooxygenases involved, StyA and StrB (flavin- and NADH-dependent). As reported by Schmid et al. the second phase acts as reservoir of the substrate and also as an *in situ* extraction of the product [43]. The yield and also the ee of the epoxidation are comparable to the epoxidation by whole cells.

Alternatively, monooxygenases can transform racemic bicyclo[3.2.0]hept-2-en-6-one to chiral lactones and thioanisole (methyl phenyl sulphide) into its chiral (*R*)-sulphoxide [45,46]. In this case, coimmobilization of the enzyme and the cofactor is necessary to keep the biocatalyst active in the organic reaction media, e.g. on Eupergit C [45]. However, it is also possible to use a membrane reactor for removing the product continuously from the reaction media. Hilker et al. reported an *in situ* substrate feeding/product removal for the Baeyer–Villiger oxidation process catalyzed by cyclohexanone monooxygenase (CHMO) (Figure 33.9) [46].

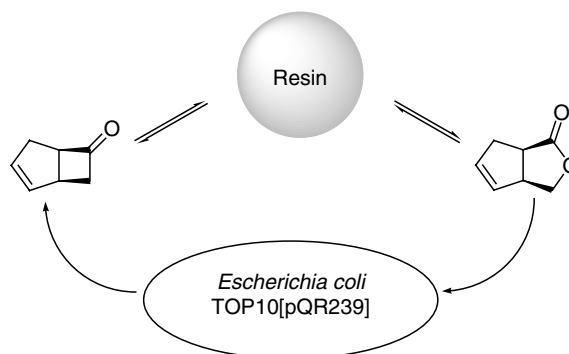


FIGURE 33.9 Regiodivergent Baeyer–Villiger oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one. (From Hilker, I. et al., *Org. Lett.*, 6, 1955–1958, 2004.)

Following this methodology they adsorbed substrate and, after reaction, also the product on a resin. Controlled by the adsorption/desorption equilibrium, the concentration of substrate and product in the aqueous bulk phase, containing enzyme and cofactor, is low. Thereby an inhibition caused by either the substrate or the product is minimized without stabilization of the biocatalyst. This approach has been first described by Zmijewsky et al. [47,48].

A functional P450cam monooxygenase was created in water–oil (w/o) emulsion formed with tetraethylene glycol dodecyl ether as a surfactant [49]. This can be used alternatively to the capsulation on a support, because the inner aqueous compartment of the w/o emulsion provides the monooxygenase with a cell-like environment in the organic bulk phase. Thus, higher efficiency and productivity of the biocatalyst can be achieved.

Many of the reaction systems that are possible (Figure 33.1) have not been reported for monooxygenases as yet. It might be effective to study the conversion of different substrates in the gas phase, because one of the reactants, oxygen, is gaseous.

In this chapter the possibilities of different reaction systems for the catalysis of oxidoreductases have been shown by some examples. Oxidoreductases are, in addition to the hydrolases, the most important EC group for industrial processes, because they are available in a large scale. It could be revealed in this chapter that the oxidoreductases are able to catalyze conversions of many different substrates in various reaction systems just like two-phase systems. In the future it would be interesting to have a look at CLEAs as a promising immobilization method [37]. However, it should be kept in mind that oxidoreductases are often used in whole-cell processes adding different requirements to the reaction system [168].

33.3 EC 2: TRANSFERASES

Transferases catalyze the transfers of functional residues of various substrates. They are divided into nine subclasses, shown in Table 33.2 [50].

The utility in organic synthesis for transferase-catalyzed reactions in nonconventional media is very low, compared to their catalytic importance in the living organism [51]. Nevertheless, several reports of organic solvent effects on transferases have been published in the past. An early example deals with the effect of the organic solvents ethanol and 1,4-dioxane on a citrate synthase from pig heart [52]. For both solvents it has been found

TABLE 33.2
Subclasses of the Transferases

EC 2 Transferases	
2.1	Transferring one-carbon groups
2.2	Transferring aldehyde or carbon groups
2.3	Acytransferases
2.4	Glycosyltransferases
2.5	Transferring alkyl or aryl groups, other than methyl groups
2.6	Transferring nitrogenous groups
2.7	Transferring phosphorous-containing groups
2.8	Transferring sulphur-containing groups
2.9	Transferring selenium-containing groups

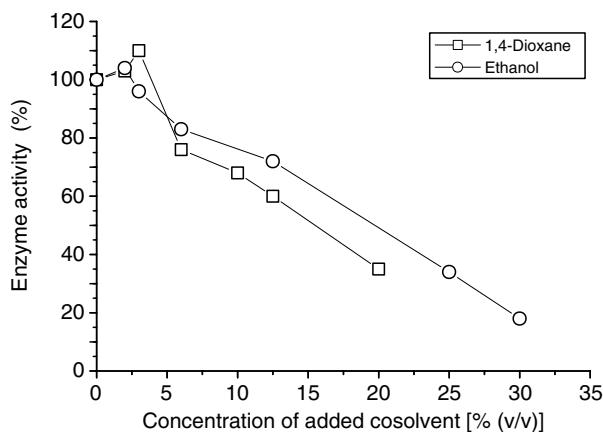


FIGURE 33.10 Effect of 1,4-dioxane and ethanol on citrate synthase from pig heart.

that with increasing amount of added solvent the enzyme activity decreases (Figure 33.10). The 50% inhibition was found to be at 16% dioxane and 19% ethanol.

In 1979 Singh and Wang reported the effects of organic solvents on a glycogen phosphorylase kinase from rabbit skeletal muscle [53]. It was observed that several organic solvents stimulate the enzyme up to a 28-fold enhancement (acetone). An overview is given in Table 33.3.

Screening the enzyme activity in correlation with increasing concentration of ethanol, a complex behavior has been observed. With an increasing amount of ethanol a relatively sharp maximum at 1.72 M was found, yielding in a kinase activity above 4 units/mg. This can be explained by ethanol-caused modification of the affinity of the protein phosphorylase b toward the kinase, which can also be done by a modification of the pH. At this point the pH-activity profile can also be modified by the organic solvent ethanol (Figure 33.10).

As shown in Figure 33.11 the activating effect is also transferable to other pH values, resulting in an activating effect. With an increase of pH the activation became negligible above a pH of 8.5; however, the causes for the behavior are not known. The authors were also able to show that all the observed effects caused by ethanol are mostly reversible, diluting out the ethanol. Therefore, the origin of the observed effects is not completely known, because no kinetic data were available.

TABLE 33.3
Enhancement of Phosphorylase Kinase–Catalyzed
Synthesis Effected by Various Organic Solvents at 1 M
Each (Unactivated Enzyme)

Solvent	Stimulation (-fold)
None (buffer)	1.0
Methanol	3.3
Ethanol	8.8
2-Propanol	18.6
Acetone	27.5
Dimethyl sulfoxide	17.8
Tetrahydrofuran	18.6

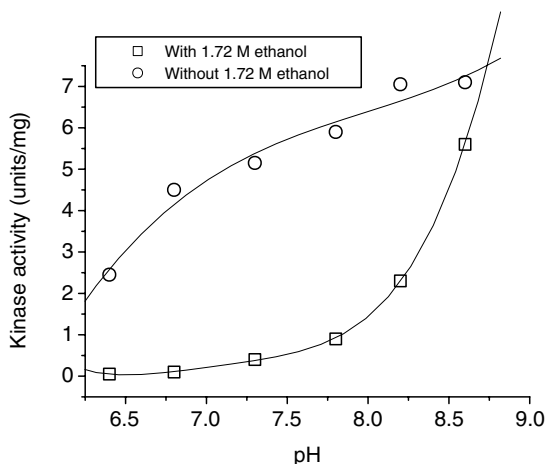


FIGURE 33.11 Ethanol-induced modification of pH dependence of (unactivated) kinase activity.

Contrarily to this, an influence of ethanol on the enzyme kinetics has been investigated for a myristoyl CoA:protein *N*-myristoyltransferase (NMT) [54]. For this transferase similar results were obtained following addition of the organic solvents, in this case for ethanol and acetonitrile (Figure 33.12).

For both added organic solvents it has been found that with increasing amount of organic solvent the NMT activity rises. After a certain amount the activity decreases again, probably because of the inactivation of the enzyme through the organic solvent.

It is noteworthy that at 10% (v/v) ethanol the NMT activity increased nearly fivefold, but the cause of this activation has yet to be found in the altered enzyme kinetic constant. This has been observed at pp60^{scr} (a peptide substrate from NH₂-terminal sequences), which showed nearly a fivefold rise in the V_{\max} value during an additional ethanol concentration of 10% (v/v) at a constant K_M value (Table 33.4).

It has been presumed that this activation is caused by a time-and-concentration-dependent unfolding mechanism. So an interference of the organic solvent molecules that arises from their different dielectric constants could lead to secondary effects on the counter-

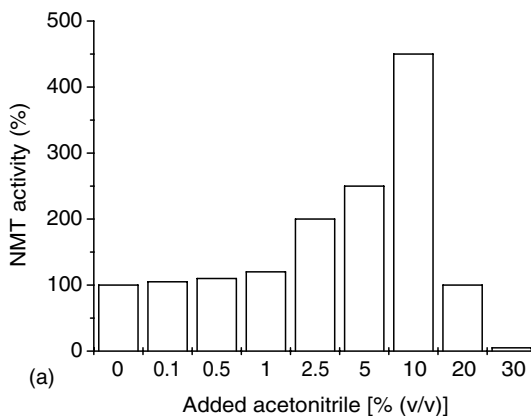


FIGURE 33.12 Effect of (a) acetonitrile and

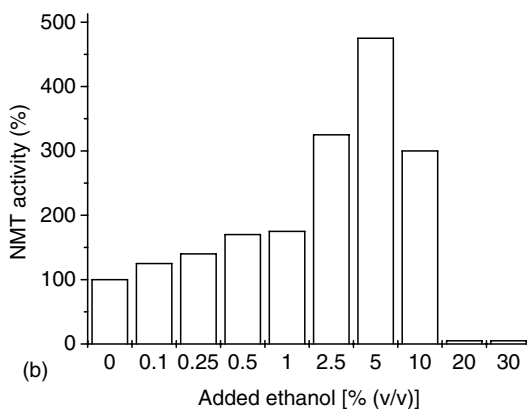


FIGURE 33.12 (continued) (b) ethanol on *N*-myristoyltransferase (NMT).

TABLE 33.4
Effect of Ethanol at Kinetic Constants

Substrate	Ethanol [% (v/v)]	K_M (μM)	Relative V_{\max} ^a
pp60 ^{src}	10	41.6	100
pp60 ^{src}	0	41.0	21.4

^a V_{\max} values are reported as a percentage of velocity observed with pp60^{src}-derived peptide substrate at 100 to 0% (v/v) ethanol.

ion atmosphere and then to the binding of the substrate [55]. Final arguments for explaining the observed behavior have not been found yet.

In contrast, transaminases are very useful catalysts for amino acid synthesis, but are generally more complex and require special expertise compared to proteases and lipases [51].

The possibility to overcome product inhibition for a ω -transaminase has been investigated by applying one- and two-phase systems [56]. Several organic solvents were tested for the enzymatic resolution reaction of α -methylbenzylamine (α -MBA), but ethyl acetate and cyclohexanone as organic solvents yielded best results for enzyme activity and biocompatibility.

The usage of a two-phase system (Figure 33.13) for transaminase-catalyzed reaction has the major advantage of an *in situ* substrate and product removal from the organic phase. Due to this the acetophenone concentration is kept at a very low level, preventing product inhibition and leading to high reaction rates.

Additionally, the easy recovery of the chiral amine can be accomplished by adjusting the pH of the aqueous phase.

The phase ratio, expressed as the volume fraction of organic phase, can also lead to much higher reaction rates (Figure 33.14). Compared with the standard system (aqueous system), the reactivity increases ninefold at a value of 0.2, which is a further advantage of the two-phase system.

In analogy to hydrolytic enzymes like subtilisin that are also used for enzymatic resolution of racemic amines, transaminases are of minor interest in nonaqueous reaction systems [51].

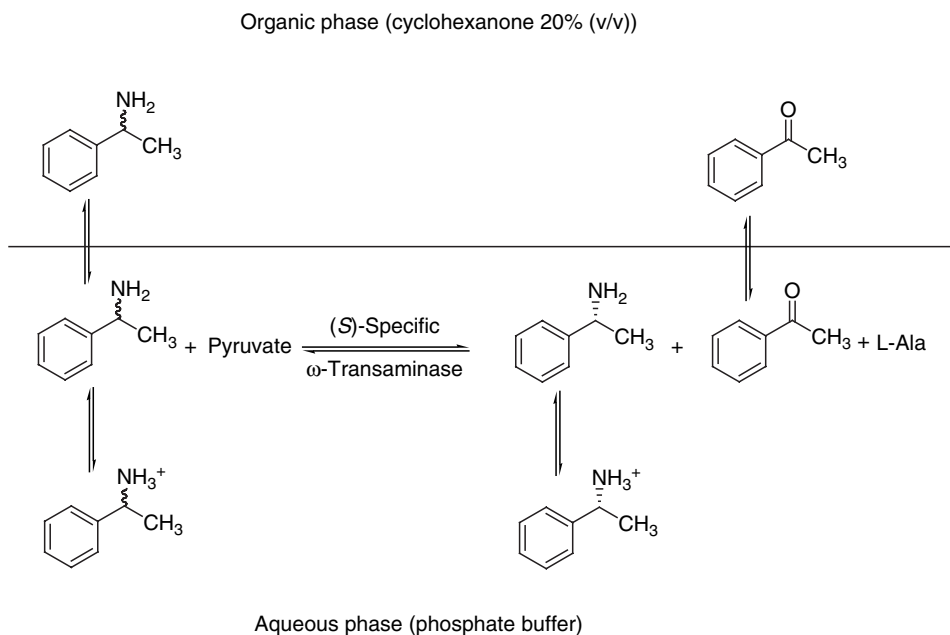


FIGURE 33.13 Two-phase-catalyzed enzymatic cleavage of α -methylbenzylamine using a ω -transaminase.

33.4 EC 3: HYDROLASES

The hydrolase family is classified in EC 3 and represents a group of enzymes that catalyzes bond cleavage by reaction with water. Several advantageous characteristics of these hydrolytic enzymes give them a high biotechnological potential and make them of special interest particularly with regard to their application in organic chemistry: (i) lack of sensitive cofactors; (ii) broad substrate specificity; (iii) high stereoselectivity; (iv) catalysis of several related reactions, such as condensation and alcoholysis; and (v) commercial availability [57].

Within the group of hydrolases, lipases (EC 3.1.1.3) stand amongst the most important biocatalysts, carrying out novel reactions in both aqueous and nonaqueous media. This is

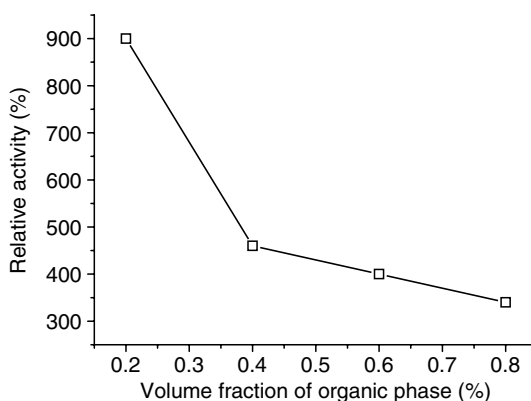


FIGURE 33.14 Enzyme activity in contrast to volume fraction of organic phase.

TABLE 33.5
Important Areas of Industrial Applications of Lipases

Industry	Examples of Use
Food industry	Bakery, dairy: flavor improvement, transesterification of fats and oils, hydrolysis of milk fat [140,141]
Paper and wood industry	Bleaching of wood and recovered paper [142]
Pharmacy	Synthesis of chiral intermediates with high enantiomeric excess [143,144]
Medicine	Application in biosensors for identification of specific lipids (diagnostic of cardiovascular diseases) [145]
Detergent industry	Cleavage of fats in laundry [141,146]
Cosmetic industry	Surfactant synthesis and flavor synthesis [147]
Environment	Wastewater treatment, conditioning of waste fat and oils [148]
Agricultural economy	Pesticide synthesis [149]
Chemistry	Polyethylene terephthalate (PET) synthesis, bioconversions, separation of enantiomers, oleochemistry [59,140,150–153]
Oleochemistry	Regioselective hydrolysis, transesterification, enantioselective processes; biodiesel, lubricants [141,154]

primarily due to their ability to utilize a wide spectrum of substrates, their high stability toward extremes of temperature, pH, and organic solvents, and their chemo-, regio-, and enantioselectivity. Lipases catalyze a wide range of reactions including hydrolysis, interesterification, alcoholysis, acidolysis, esterification, and aminolysis. They are used in a variety of biotechnological fields such as food and dairy, detergent, and pharmaceutical, agrochemical, and oleochemical industries (Table 33.5). Lipases can be further exploited in many newer areas where they can serve as potential biocatalysts. Due to the tremendous potential of lipases for exploitation in biotechnology this section will mainly review this group of hydrolases.

Lipases are ubiquitous in nature: they occur in plants, animals, and microorganisms, and are primarily responsible for the hydrolysis of triglycerides with concomitant production of free fatty acids and glycerol. Many lipases are excreted extracellularly by fungi and bacteria, which makes their large-scale production particularly easy. In addition to their specific and limited function in metabolism, lipases play an important role in biotechnology: about 40% of all biotransformations reported so far have been performed with lipases [51]. In general, lipases are characterized by their unique feature of acting at water–organic interfaces, which distinguishes them from esterases [58,59]. Research in this field suggests that a lipid-induced conformational change alters the orientation of a lid that covers the enzyme active site. This phenomenon is commonly known as interfacial activation [57]. Consequently, lipase-catalyzed reactions are preferably conducted in two-phase systems.

Bearing in mind that the natural substrates of lipases are esters of an alcohol—glycerol—with an achiral acid, it is understandable that lipases are particularly useful for the resolution or asymmetric esterification of esters bearing a chiral alcohol moiety. On the basis of a thorough survey of the literature on chiral resolutions with lipases from *Candida rugosa* (CRL) and *Pseudomonas cepacia* (PCL), Bornscheuer and Kazlauskas proposed rules for the enantioselectivity of these two enzymes on the spatial requirements of the substituents on the reagent [57]. The basics of “Kazlauskas rules” are shown in Figure 33.15 and literature has shown this rule to be highly predictive for lipase action on secondary alcohols, but less accurate for lipase-catalyzed transformations of primary alcohols and acids.

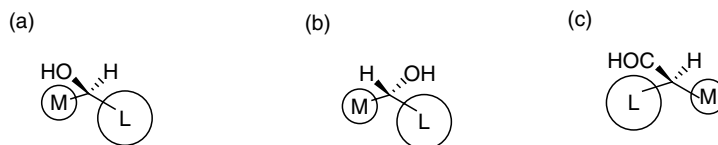


FIGURE 33.15 Scheme of the “Kazlauskas rule” to predict which enantiomer of (a) a secondary alcohol, (b) a primary alcohol, and (c) a carboxylic acid reacts faster in lipase-catalyzed reactions. M, medium-sized substituent, L, large-sized substituent.

Commercial lipase from *C. rugosa* is probably the most often used biocatalyst. This lipase has been applied for several selective hydrolysis reactions of esters of cyclic secondary alcohols, because its active site accepts larger substrates than those of other lipases [57]. One example to illustrate this point is the resolution of racemic 2,3-dihydroxy carboxylates and cyclohexane-1,2,3-triol esters by CRL [60,61]. PCL possesses a “smaller” active site than CRL and can be extremely selective on “slim” counterparts as was shown for the desymmetrization of some prochiral dithioacetal esters [62–66].

During the past decades the general opinion about the use of enzymes in organic reactions has changed. Conventional biocatalysis had mainly been performed in aqueous solutions, because enzymes were considered to be most active in water—their natural milieu. Subsequent years have seen the replacement of this prejudice by a more refined position initiated by the discovery that many enzymes are active in organic media.

Publications from the early 20th century and the 1930s already reported about biocatalysis in organic media, but most of this work was forgotten [67]. About 60 years ago researchers published first results about lipase-catalyzed esterifications in organic solvents containing approximately 8 to 12% of water [68,69]. However, it took more than 40 years to convince researchers that enzyme-catalyzed reactions are not only possible but also sometimes more convenient in organic solvents. Since the pioneering work of Cambou and Klivanov on this subject many publications have appeared on enzyme-catalyzed kinetic resolutions in organic solvents (especially lipase-catalyzed reactions) and on related topics like the use of organic cosolvents in water, reactions in solvent-free media or in compressed or supercritical gases, and the addition of salts or (thia)crown ethers to the lipase [70].

When performing lipase-catalyzed reactions in organic media, the ability to do an esterification reaction instead of hydrolysis is one advantage. It has been found that although lipases favor the same prochiral group in both cases, the two reactions yield opposite enantiomers. Figure 33.16 gives an example of the phenomenon: the acetylation of 2-benzylglycerol with PCL yields the (*S*)-monoacetate, while hydrolysis of the diacetate with porcine pancreatic lipase (PPL) yields the (*R*)-monoacetate.

Another advantage results from potential changes in the selectivity of lipases by applying different solvents, sometimes called medium or solvent engineering. Changes in the

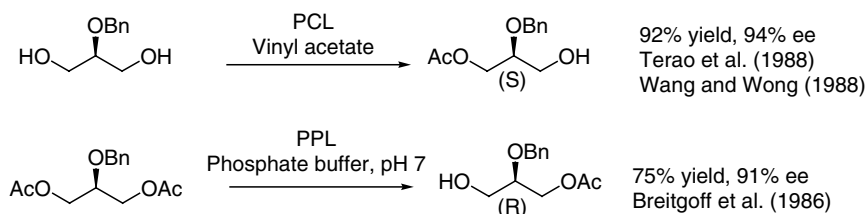


FIGURE 33.16 Acetylation of benzylglycerol with lipase of *Pseudomonas cepacia* (PCL) and hydrolysis of the diacetate with porcine pancreatic lipase (PPL).

enantioselectivity after varying the solvent could be shown for PPL. Mori et al. found no enantioselectivity for the hydrolysis of seudenol acetate, but Johnston et al. reported moderate enantioselectivity ($E = 17$) in the acetylation of seudenol with trifluoroethyl acetate in ethyl ether [71,72]. These are only a few examples from the large volume of literature on lipase-catalyzed reactions in organic solvents. In order to give an overview of other techniques applied for these reactions the following sections will briefly discuss recent developments in other reaction systems.

33.4.1 IMMOBILIZATION

Since the second half of the 20th century, investigations have focused on the development of immobilized enzymes in order to improve process economy by allowing the continuous or repetitive use and easy recovery of enzymes. Immobilization refers to the preparation of insoluble derivatives of enzymes, and has been performed by various methods including (i) noncovalent adsorption or deposition; (ii) covalent attachment; (iii) entrapment in a polymeric gel, membrane, or capsule; and (iv) cross-linking of the enzyme [73]. These techniques can dramatically affect enzyme properties such as pH dependence, temperature profile, and kinetics, and have often resulted in biocatalysts exhibiting significantly higher stability than the native enzyme.

Back in the 1950s, first immobilization methods for lipases have been reported. Although enzyme immobilization was dominated by physical methods during this time, first results of specific ionic adsorption like the binding of lipase on styrenepolyaminostyrene (Amberlite XE-97) have been published [74]. Unfortunately, those early-developed carriers were found to be not very suitable for enzyme immobilization. Since then many types of enzymes immobilized by different immobilization techniques have been found to exhibit higher stabilities, activities, and/or selectivities than native enzymes. Regarding lipases, entrapment in alkyl-substituted organic silane precursors has led to increased activity of these enzymes in organic solvents. The obtained sol-gels even show a significantly higher activity than freely dispersed enzymes [75]. The development of a novel technique for the immobilization of lipases by entrapping the enzymes within an aqueous solution in bead-shaped silicone elastomers ("static emulsion") led to an enhancement in enzyme activity by factor 31 for *C. antarctica* lipase A (CAL-A) and factor 250 for *Thermomyces lanuginosa* lipase in comparison to the native enzyme in hexane [76]. By entrapment of lipase-lipid complexes in *n*-vinyl-2-pyrrolidone gel matrix, Goto et al. succeeded in increasing the activity up to 50-fold in comparison to the native enzyme [77].

A variety of immobilization techniques like covalent bonding, entrapment, and adsorption can be applied to change the selectivity of lipases. For example, the *S*-selective lipase from *C. rugosa* presented a high enantioselectivity ($E = 400$) toward the *R*-isomer for the resolution of mandelic acid esters after covalent immobilization on glutaraldehyde supports [78].

In recent years, carrier-bound CLEAs have attracted increasing attention, due to their simplicity, broad applicability, high stability, and high volume activity. CLEAs of CRL with enhanced activity, stability, and defined particle size have been designed by impregnation of enzyme solution in a porous membrane of controlled pore size followed by subsequent aggregation and cross-linking [79].

33.4.2 IONIC LIQUIDS

Over the last decade ILs have emerged as alternative reaction media for performing all types of reactions with sometimes remarkable results [80–82]. In 2000, Lau et al. investigated the reactivity of *C. antarctica* lipase B (CAL-B) in ILs, such as [BMIM][PF₆] and [BMIM][BF₄],

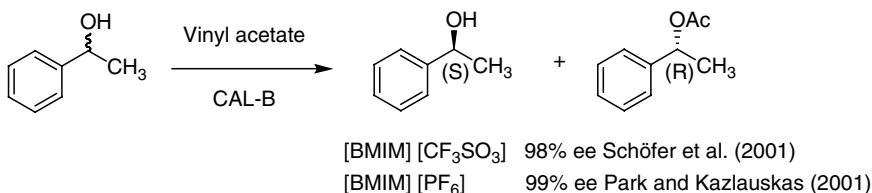


FIGURE 33.17 Lipase-catalyzed reaction in the presence of ionic liquids (ILs).

in comparison to conventional organic solvents [83]. They found similar reaction rates for all the reactions investigated. This work represents the second publication to demonstrate the potential use of ILs for enzyme catalysis and the first to show their use with lipases.

Since then several other lipases have been applied to catalyze reactions in systems with ILs as pure solvents, and as cosolvents with water or in two-phase systems. For example, lipase-mediated kinetic resolution of racemates in ILs provides extremely high enantiopurities of the products [84–86]. Kragl et al. investigated the kinetic resolution of 1-phenylethanol for a set of eight different lipases in ten different ILs with MTBE as reference [84]. To investigate transesterification vinylacetate was used as acetyl donor. The best results were obtained for CAL-B in [BMIM][CF₃SO₃], [BMIM][(CF₃SO₂)₂N], and [OMIM][PF₆]. Park and Kazlauskas reported good activities for CAL-B by using the ILs [BMIM][BF₄] and [BMIM][PF₆] in the same system (see Figure 33.17) [86].

Moreover, CAL-B has been found to exhibit impressive regioselectivities for the C-6 monoacetylation of β -D-glucose in [MOEMIM][BF₄]. Due to their solvation properties, ILs dissolve not only hydrophobic compounds but also hydrophilic compounds such as carbohydrates. Park and Kazlauskas reported the regioselective acylation of glucose with 99% yield and 93% selectivity in [MOEMIM][BF₄] (Figure 33.18). These values are much higher than those observed in the organic solvents commonly used for this purpose (reaction carried out in acetone: 72% yield, 76% monoacetylation) [86].

33.4.3 REVERSE MICELLES

The low water content necessary to favor synthesis reactions in organic media by lipases can be achieved by microencapsulation of the biocatalyst within reverse micelles. Reverse micelles consist of tiny aqueous droplets stabilized by surfactants in a bulk water-immiscible organic solvent. The biocatalyst remains soluble and active in the water phase, while reacting with water-insoluble or poorly soluble compounds present in the organic phase. The reversed micellar system has been proven to be highly suitable for lipase-catalyzed reactions. As the amount of aqueous phase is very small, lipases can catalyze transesterification and ester synthesis. Moreover, the system provides a high interfacial area and thus eliminates mass

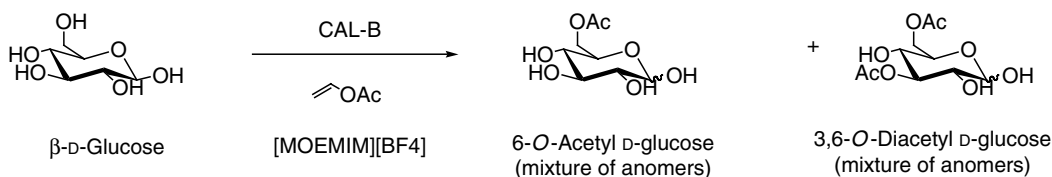


FIGURE 33.18 The acylation of glucose using *Candida antarctica* lipase B (CAL-B) in the ionic liquid (IL) [MOEMIM][BF₄]. (From Park, S. and Kazlauskas, R.J., *J. Org. Chem.*, 66, 8395–8401, 2001.)

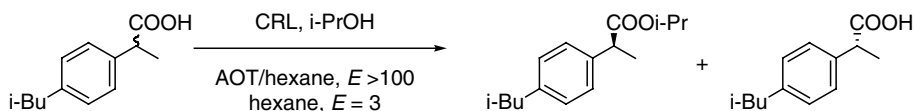


FIGURE 33.19 Esterification of ibuprofen by *Candida rugosa* lipase (CRL). (From Hedstrom, G., Backlund, M., and Slotte, J.P., *Biotechnol. Bioeng.*, 42, 618–624, 1993.)

transfer limitations. Many surfactants and solvents can be applied, but anionic surfactants, in particular AOT, have been proven to be best in lipase-catalyzed reactions [87].

The application of lipases in reverse micelles results in small changes in enzyme selectivity. In 1987 Bello et al. investigated the selectivity of CRL in the transesterification of triglycerides in reversed micelles and reported that CRL, which normally shows little fatty acid chain length selectivity, favored longer chain lengths in this system [88]. Moreover, the enantioselectivity of CRL can be increased by applying the biocatalyst in reverse micelles. Hedström et al. reported enantioselectivities of $E > 100$ for the CRL-catalyzed esterification of ibuprofen in reversed micellar systems in comparison with enantioselectivities of $E = 3$ in hexane (Figure 33.19) [89].

Many other applications of lipases in reverse micellar systems can be found in literature. Table 33.6 shows selected examples of reactions catalyzed by lipases with potential applications in food, pharmaceutical, and chemical industries as well as in the environmental area.

However, the recovery of products from surfactant-containing organic solvents still represents a problem that must be overcome before the reverse micellar system can be effectively applied at industrial scale. One possible solution might be the continuous operation in membrane reactors: an ultrafiltration membrane can be used to retain the micelles while the small molecules of substrate and products pass freely [90].

33.4.4 SUPERCRITICAL FLUIDS

Lipase-catalyzed reactions have proven to be feasible in supercritical fluids (scF). These fluids represent substances heated above their critical temperature and compressed above their critical pressure. They exhibit properties similar to those of hydrophobic solvents, show

TABLE 33.6
Selected Examples of Reactions Catalyzed by Lipases in Reversed Micellar Systems

Enzyme/Source	System (Surfactant/Organic Solvent)	Reaction
<i>Thermomyces lanuginosa</i>	AOT Isooctane	Synthesis of ethyl-laurate [155]
<i>Bacillus megaterium</i>	AOT <i>n</i> -Heptane	Hydrolysis of <i>p</i> NPP [156]
<i>Rhizopus delemar</i>	AOT Isooctane	Hydrolysis of triolein [157]
<i>Mucor javanicus</i>	AOT Isooctane	Acylation of doxorubicin [158]
<i>Candida lipolytica</i>	AOT Isooctane	Esterification of octanoic acid with 1-octanol [159]

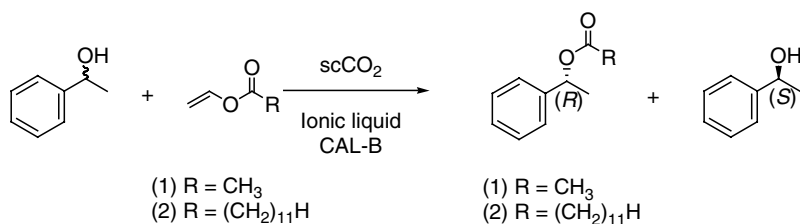


FIGURE 33.20 Lipase-catalyzed kinetic resolution in scCO_2 . (From Reetz, M.T. et al., *Adv. Synth. Catal.*, 345, 1221–1228, 2003.)

rapid mass transfer due to low viscosity, and allow a simple downstream processing by evaporation. Moreover, their solvation properties can be changed by changing the pressure.

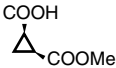
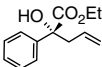
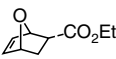
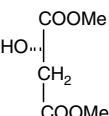
The use of supercritical carbon dioxide is probably the most common way to conduct enzyme-catalyzed reactions in scFs, because it is nonflammable, nontoxic, cheap, and reaches the supercritical state at 31.1°C . The first application of lipase-catalyzed reactions in scFs dates back to 1986. Nakamura et al. reported about the *Rhizopus oryzae* lipase (ROL)-catalyzed interesterification of triolein and stearic acid to 8% conversion in scCO_2 [91]. Since then researchers have examined a wide range of reactions and observed changes in conversion, enantioselectivity, and lipase stability similar to those in organic solvents. For example, Reetz et al. found high enantioselectivities for the lipase-catalyzed kinetic resolution of chiral racemic secondary alcohols (Figure 33.20) [92]. Lozano et al. and Reetz et al. reported the immobilization of CAL-B in ILs (Section 34.4.5), whereas substrates and products are dissolved in a second phase formed by supercritical CO_2 [93,94]. Further examples for lipase-catalyzed reactions in scFs can be seen in Table 33.7.

To summarize, this chapter aims to deliver a short insight into the immense possibilities of lipase application in organic synthesis. Future research will focus on the development of a new generation of lipases by extensive screening and genetic manipulations in order to create task-specific lipases for special applications. Protein-engineering methods will help to make

TABLE 33.7
Reactions in Supercritical Fluids (scFs) Catalyzed by Lipases

Enzyme/Source	System (Surfactant/Organic Solvent)	Reaction
Free and immobilized lipases: <i>Rhizomucor meihei</i> , <i>Pseudomonas fluorescens</i> , <i>Rhizopus javanicus</i> , <i>R. niveus</i> , <i>Candida rugosa</i>	scCO_2 and scPropane	Ester synthesis: oleyl oleate [160]
Free and immobilized lipases: <i>C. antarctica</i> , <i>Mucor miehei</i>	scCO_2 and ionic liquids	Synthesis of glycidyl esters: kinetic resolution of <i>rac</i> -glycidol [161]
<i>C. antarctica</i> lipase (Novozyme 435)	scMethanol , scEthanol , and scCO_2	Synthesis of biodiesel [162]
Immobilized lipase: <i>M. miehei</i>	scCO_2	Hydrolysis of blackcurrant [163]
Immobilized lipase: <i>C. antarctica</i>	scCO_2	Butyl butyrate synthesis [164]

TABLE 33.8
Selected Pig Liver Esterase (PLE)-Catalyzed Reactions

Reaction	Substrate	Enzyme Source
Asymmetrization of nonchiral substrates [98]		PLE
Optical resolution of racemates [165]		PLE
Separation of endo/exoisomers [166]		PLE
Regioselective hydrolysis of ester groups [167]		PLE

lipases versatile industrial biocatalysts by enabling the production of large amounts of recombinant enzymes and the improvement of their biochemical and catalytic features.

33.4.5 OTHER HYDROLYTIC ENZYMES

Esterases (carboxylester hydrolases, EC 3.1.1.1) catalyze, like lipases, the hydrolysis of carboxylic acid esters but, in contrast to lipases, only a few esterases have practical use in organic synthesis. Most of the esterase-catalyzed reactions in literature have been performed by the esterase isolated from pig liver (PLE) [95]. In contrast to lipases, PLE shows highest activity in aqueous buffered or two-phase systems, and usually does not accept highly hydrophobic substrates. Thus, selectivity tuning is more or less limited to the addition of up to 20% of polar protic water-miscible cosolvents like methanol, *tert*-butanol, DMSO, acetone, or acetonitrile [96]. In 1997 Ruppert and Gais succeeded to enhance the activity of PLE in organic solvents after colyophilization of the esterase with methoxypolyethylene glycol [97].

Esterases are in general very useful biocatalysts for the production of chiral intermediates through hydrolysis reactions. Examples include the asymmetrization of prochiral substrates and the optical resolution of racemates [98,99]. Esterases have also been used to separate endo/exo-mixture and for the regioselective hydrolysis of an ester group in the presence of a second ester function (Table 33.8).

Proteases are the last group of hydrolases to be mentioned in this review because they represent one of the three largest groups of industrial enzymes and find application in detergents, leather, food, and pharmaceutical industries, as well as bioremediation processes [100,101].

The most important commercial proteases are subtilisin, α -chymotrypsin, and—to a lesser extent—trypsin, pepsin, papain, and penicillin acylase. In the field of organic synthesis, two main applications of proteases can be found: (i) the enantioselective hydrolysis of carboxylic acid esters, where they seem to retain a preference for the hydrolysis of that enantiomer, which mimics the configuration of an L-amino acid more closely; and (ii) the synthesis of di- and oligopeptides by coupling of *N*-protected amino acids and peptide esters. The latter has been carried out on an industrial scale by Tosoh Corporation (Japan) for the thermolysin catalyzed synthesis of aspartame (Figure 33.21) [102,103].

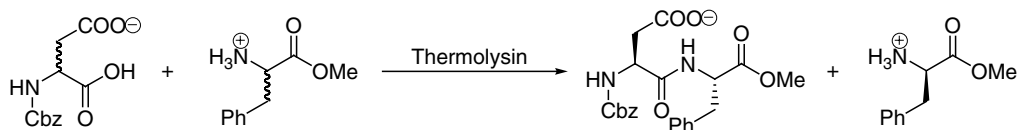


FIGURE 33.21 Commercial process for the production of aspartame by Tosoh Corporation (Japan).

TABLE 33.9
Subclasses of the Lyases

EC 4 Lyases	
4.1	Carbon–Carbon lyases
4.2	Carbon–oxygen lyases
4.3	Carbon–nitrogen lyases
4.4	Carbon–sulfur lyases
4.5	Carbon–halide lyases
4.6	Phosphorous–oxygen lyases
4.99	Other lyases

33.5 EC 4: LYASES

Apart from oxidoreductases and hydrolases, lyases are the most frequently used enzyme class [51]. They are divided into several subgroups, which are shown in Table 33.9 [50].

These enzymes catalyze several bond formation and cleavage reactions and have found extensive usage in several large-scale applications [104]. Two important examples, shown in Figure 33.22—(a) acrylamide synthesis and (b) *N*-acetylneuraminic acid—illustrate their use in the synthesis of bulk and fine chemicals.

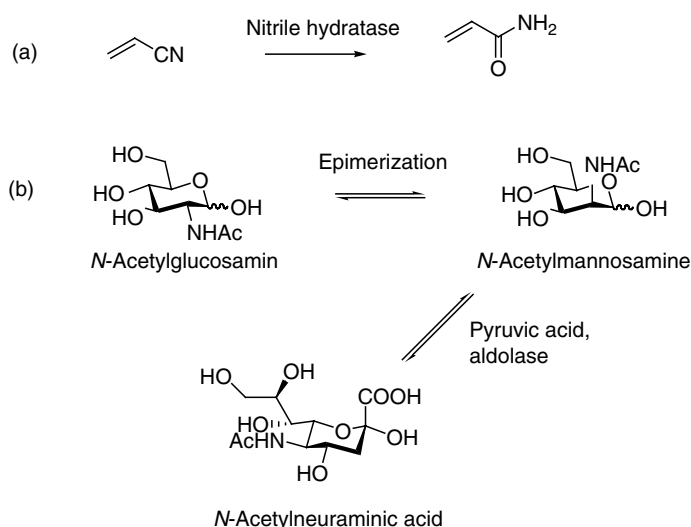


FIGURE 33.22 Large-scale applications of lyases. (From Wandrey, C., Liese, A., and Kihumbu, D., *Org. Proc. Res. Dev.*, 4, 286–290, 2000.)

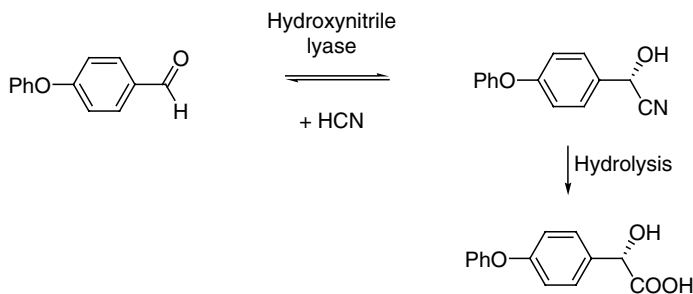


FIGURE 33.23 α -Hydroxy-carboxylic acids synthesis using hydroxynitrile lyases.

The cyanohydrin formation using hydroxynitrile lyases (HNLs) from several sources has produced enormous interest in the last decade. Important follow-up products are α -hydroxy-carboxylic acids, which are easily accessible using these enzymes (Figure 33.23), and have also been applied in large-scale operations [105].

33.5.1 NITRILE HYDRATASES

Nitrile hydratases hydrolyze nitriles selectively to the resulting amides. An important example is the enzymatic formation of acrylamide, see also Figure 33.24. The amide can be converted afterwards to the corresponding carboxylic acid. This two-step approach can also be simplified by using a nitrilase (Figure 33.24), yielding in one step the desired carboxylic acid [51].

In amide synthesis, using a nitrile hydratase, an important factor is the stability of the biocatalyst related to the rising product concentration [$>50\%$ (w/v) acrylamide], causing a rapid enzyme deactivation [106].

Several nitrile hydratases have been adapted to these challenges in acrylamide synthesis, e.g., the nitrile hydratase from *Rhodococcus* sp. N-774 in 1987 [107,108]. In 1988 a much more stable enzyme has been purified from *P. chloroaphis* B23 that exhibited a higher stability and reactivity in synthesis. An even more resistant nitrile hydratase from *Rhodococcus rhodochrous* J1 has been found in 1993. This enzyme exhibited an enormous stability against a variety of organic solvents (Table 33.10) [106].

As shown in Table 33.10, the variant from *R. rhodochrous* exhibits the highest stability against the organic solvents and also an activating effect for ethylene glycol. It has been assumed that the outstanding stability derives from the very high molecular mass of 505 kDa (20 subunits), by suppressing the flexibility of the protein.

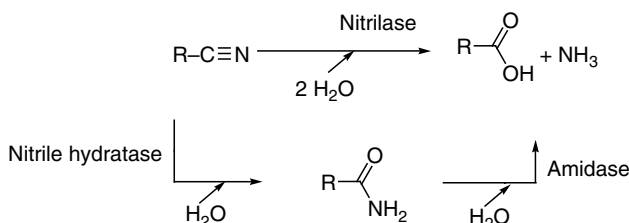


FIGURE 33.24 General pathway of the enzymatic hydrolysis of nitriles.

TABLE 33.10
Nitrile Hydratase Stability against Various Organic Solvents (Abstract)

Organic Solvent (50% (v/v))	Relative Activity (%)		
	<i>Brevibacterium</i> R312	<i>Pseudomonas</i> <i>chlororaphis</i> B23	<i>Rhodococcus</i> <i>rhodochrous</i> J1
None	100	100	100
Methanol	6	8	89
Ethanol	8	10	94
Acetone	10	12	66
Dimethyl sulfoxide	22	38	58
Ethylene glycol	64	55	136

In the past few years an enormous number of new nitrile-converting enzymes have been discovered, possessing high enantioselectivities and broad substrate ranges [109]. Nitrilases have also been successfully applied to organic solvents, like *Pseudomonas* sp. DSM 11387, as shown in Table 33.11 [110].

It can be seen that the relative activity is rising with increasing log *P* value, an observation that is comparable with other enzymes [111].

In the future, new immobilization techniques will provide even more stable enzymes. First attempts using the technique of CLEAs have been successfully arranged for nitrilases [37]. Additionally, the search for new enzyme sources and the modification of established nitrile-converting enzymes could lead to higher reactivities and selectivities. Potentially the combination of HNLs for obtaining cyanohydrins and the usage of nitrile hydratases for hydrolysis of the cyanohydrin in one step may also become an “interesting reaction” in the future [112,113].

33.5.2 HYDROXYNITRILE LYASES

The HNLs reversely catalyze the enantioselective formation and cleavage of cyanohydrins. Several HNLs are known that convert (in the synthesis reaction) a broad range of aldehydes and ketones into the corresponding (*R*)- or (*S*)-cyanohydrins (Figure 33.25 gives examples of enzyme sources) [114].

Since the early works of Rosenthaler, in which he uses the enzyme preparation *emulsin* (from almond), several other enzyme sources have been intensely studied and reviewed for the cyanohydrin syntheses [114,115]. During the 1960s and 1970s HNLs were rediscovered and used in several large-scale applications. In the last decade an intense study on these enzymes led to the enclosing overlook about mechanism and structure for nearly all known HNLs.

TABLE 33.11
Stability of a Nitrilase in Organic/Aqueous Mixtures

Organic Solvent (50% (v/v))	log <i>P</i>	Relative Activity
No addition	—	100
1-Octanol	2.9	47
Octane	4.5	66
Hexadecane	8.8	97

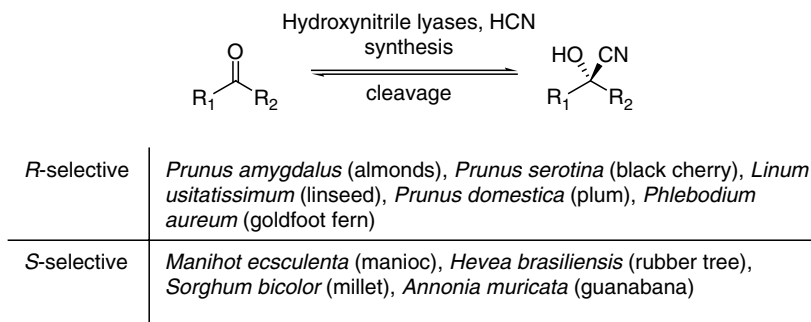


FIGURE 33.25 Hydroxynitrile lyase-catalyzed reactions.

They can be divided into FAD-dependent (e.g., *Prunus amygdalus*) and non-FAD-dependent (e.g., *Manihot esculenta*) HNLs [116].

Beginning with an emulsion of benzaldehyde in water, used by Rosenthaler, several different reaction systems have been developed. As an important example, the immobilized enzyme on various supports has to be mentioned and has found broad usability for various HNLs like the successful application to the HNL from *M. esculenta* (Table 33.12) [117–119]. The enzyme is adsorbed on nitrocellulose suspended in an organic solvent (e.g., diisopropyl-ether). Using this approach several aldehydes and ketones can be converted with high yields and very good ee [120].

Even though a macroscopic suspension (enzyme support suspended in an organic solvent) is formed, this system behaves like a one-phase system (see Section 33.1). Due to this restriction, limitations like substrate and product inhibition may occur. This disadvantage can be solved by using the very effective two-phase approach, using a buffer phase containing the enzyme and a water-immiscible organic solvent containing substrates. Applying this system the substrate and product concentration in the aqueous phase is very low, resulting from the partition coefficients between organic and aqueous phases.

Using this approach, several aldehyde and ketone cyanohydrins were easily accessible using the HNL from *Hevea brasiliensis* (Table 33.13) in a two-phase system consisting of a buffer and MTBE [121]. Recently, Lou et al. presented the first hydroxynitrile lyase-catalyzed reaction with encouraging results using the HNLs from *P. amygdalus* (*Pa*HNL) and *H. brasiliensis* (*Hb*HNL) in the IL–buffer two-phase system [122].

TABLE 33.12
Cyanohydrin Synthesis Using Adsorbed Wild-Type Hydroxynitrile Lyase from *Manihot esculenta*, Abstract

Substrate	Reaction Time (h)	Conversion (%)	ee (S) (%)
Benzaldehyde	0.5	97	99
2-Chlorobenzaldehyde	1	96	98
3-Phenoxybenzaldehyde	6.25	47	96
Decanal	17	65	78
Acetophenone	3	13	78
2-Thiophen	0.5	75	97

TABLE 33.13
Two-Phase System in Hydroxynitrile Lyase-Catalyzed Synthesis

Substrate	Water-Immiscible Solvent	Conversion (%)	ee (S) (%)
Benzaldehyde	MTBE	97	99
3-Phenoxy-benzaldehyde	MTBE	99	99
Propenal	MTBE	92	98
Benzaldehyde (<i>Pa</i> HNL)	PMIM BF ₄	99	97 (<i>R</i>)
Benzaldehyde (<i>Hb</i> HNL)	PMIM BF ₄	99	99

Furthermore, with the HNL from *H. brasiliensis* it has been demonstrated that the enzymatic reaction is performed only at the interfacial area and not in the aqueous bulk phase. This has been observed for the mandelonitrile cleavage by a blockade of the interface between diisopropylether and buffer [123].

Due to several new discovered HNLs and large-scale applications for cyanohydrins, using established enzymes, the observation of this enzyme class will proceed. Additionally, only a few reports were published using ILs in lyase-catalyzed reactions, especially for HNLs [122]. The research interest in the field of “enzymes in nonconventional media” will probably rise in the next few years. Also the usage of new molecular-biological attempts will gain more interest, with techniques like directed evolution. For example, a single residual replacement improves the folding and stability of a HNL from *M. esculenta* [125].

33.5.3 ALDOLASES

The usability of directed evolution in aldolase synthesis has been shown for a fructose biphosphate aldolase. After four rounds of directed evolution using DNA shuffling of the *fda* genes from *E. coli* and *Edwardsiella ictahuri* the resistance against various organic solvents and also the thermostability have been increased [126].

The wild-type enzyme (*E. coli*) lost 40 to 90% of activity, whereas the variant activity remained nearly constant for an additional 20% (v/v) organic solvent (Figure 33.26). For a

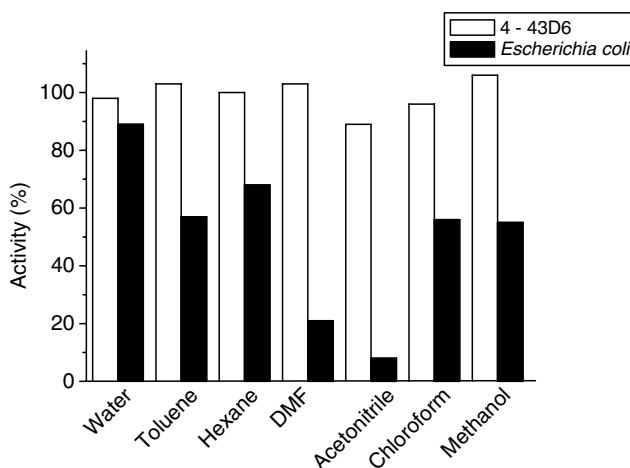


FIGURE 33.26 Irreversible inactivation, caused by the organic solvent [20% (v/v)].

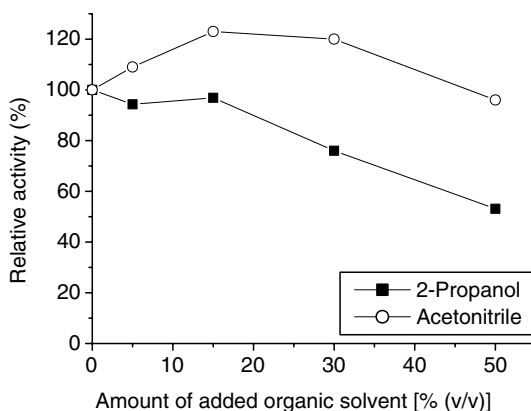


FIGURE 33.27 Influence of 2-propanol and acetonitrile on the aldolase from *Hyperthermophilic archaea*.

heat-stable aldolase from *Methanococcus jannaschii* also a slight activating effect has been observed. By an addition of 15% (v/v) acetonitrile the relative activity increased to 23%, as shown in Figure 33.27 [127].

This stability against organic solvents may also be valuable for less-soluble substrates in enzyme catalysis in organic synthesis. A rabbit muscle aldolase (RAMA) showed an impressive stability with several water-miscible organic solvents (Figure 33.28).

Only for a few water-miscible organic solvents the enzyme activity was significantly reduced, whereas the water-immiscible organic solvents showed only a low decrease (maximum 50%).

As many substrates for aldolases are well soluble in water, the use of nonconventional media will be of limited interest.

33.6 EC 5: ISOMERASES

Isomerases catalyze isomerization reactions like racemization, epimerization, and rearrangement of different substrates. The utility of such enzymes is very low in industry because, on the one hand, there are only a few commercially available and, on the other hand, the

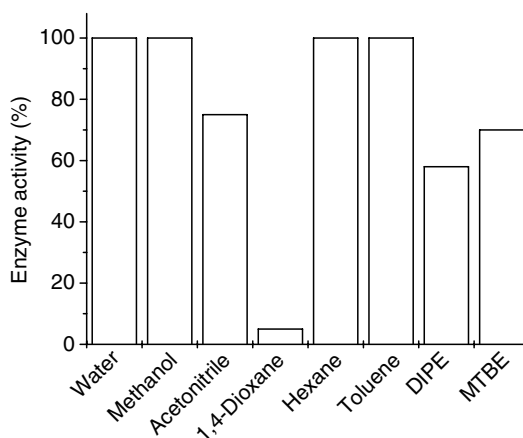


FIGURE 33.28 Rabbit muscle aldolase (RAMA) activity after 24 h of incubation at 20% (v/v), abstract.

TABLE 33.14
Subclasses of the Isomerases

EC 5 Isomerases	
EC 5.1	Racemases and epimerases
EC 5.2	<i>cis-trans</i> -Isomerases
EC 5.3	Intermolecular Oxidoreductases
EC 5.4	Intermolecular transferases
EC 5.5	Intermolecular lyases
EC 5.99	Other isomerases

exigency of isomerization in industry is marginal. Therefore, only about 2% of the publications in the period between 1987 and 2003 dealt with isomerase-catalyzed synthesis [51]. Isomerases are further divided into six subclasses (Table 33.14) [50].

In the last 25 years of the 20th century most reports were dealing with racemases and epimerases, which can be used for the dynamic kinetic resolution to get 100% conversion [51]. Yagasaki and Ozaki published the production of γ -aminobutyrate [128]. D-Glutamic acid was produced from L-glutamic acid. L-Glutamate was converted to the DL-form by the recombinant glutamate racemase of *L. brevis* ATCC8287. Then L-glutamate in the racemic mixture was selectively decarboxylated to the product by L-glutamate decarboxylase of *E. coli* ATCC11246. This was successfully realized in a one-pot reaction. Before this, there were a few articles dealing with cofactor independence of glutamate racemase from *Lactobacillus* sp. Some racemases like alanine racemase need a cofactor to catalyze the enzymatic racemization. In the case of the alanine racemase, pyridoxal phosphate (PLP) is the necessary cofactor. In addition to the relatively well-studied alanine racemase, arginine racemase from *Pseudomonas* sp. and two serine racemases from *Streptomyces* sp. require PLP for their catalytic activity [129].

The epimerization of threonine could be observed by the new amino acid racemase from *P. putida*. As mentioned earlier this epimerization has been done in buffered aqueous system as well, with attention on cofactor dependence—again the cofactor is pyridoxal 5'-phosphate—and on substrate specificity [130].

A typical enzyme to employ dynamic kinetic resolution is the mandelate racemase. In combination with a second biocatalyst, it is possible to transfer a racemate into a single stereoisomer in 100% theoretical yield [131]. Mandelate racemase catalyzes the racemization of different substrates in aqueous solution (HEPES pH 7.5 with 3.3 mM $\text{MgCl}_2 \cdot \text{H}_2\text{O}$) at room temperature, with further reaction by lipase from *Pseudomonas* sp. [132].

Syntheses catalyzed by isomerases are usually not investigated in nonconventional media. But the *O*-acylation of (\pm)-mandelic acid by lipase from *Pseudomonas* sp. was achieved in diisopropylether, while the mandelate racemase-catalyzed racemization took place in the aqueous phase [133]. Kaftzik et al. first reported the use of mandelate racemase in ILs as cosolvent and in two-phase systems (Figure 33.29) [134]. It is shown that the activity of the racemase strongly depends on the water activity of the reaction system. Activity of the mandelate racemase could be obtained in [BMIM][OctSO₄] at water activity a_w :0.75, and in biphasic systems consisting of water and [OMIM][PF₆] in a ratio of 1:10.

Isomerases will probably not find much consideration in organic synthesis taking place in nonconventional media in the future. Because of their scarce availability and limited purpose in biocatalytical synthesis, there are only a few applications.

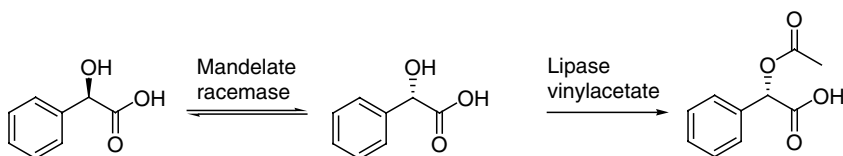


FIGURE 33.29 Deracemization of (\pm)-mandelic acid through a lipase-mandelate racemase two-enzyme system: *Pseudomonas* sp. lipase catalyzes *O*-acylation of (\pm)-mandelic acid and mandelate racemase-catalyzed racemization of remaining unreacted (*R*)-mandelic acid. (From Kaftzik, N. et al., *Mol. Catal. A Chem.*, 214, 107–112, 2004.)

33.7 EC 6: LIGASES

Ligases (synthetases) are classified in group 6 by the EC and represent a class of enzymes that catalyze the formation of bonds between two substrate molecules. They are further divided into subclasses according to the type of bond formed (Table 33.15).

The synthesis reaction catalyzed by ligases requires the hydrolysis of a nucleoside triphosphate such as adenosine triphosphate (ATP). In the field of ligases, examples of industrial biocatalysis are more or less missing [104]. This is further indicated by the fact that only about 1% of research from 1987 to 2003 has been performed with enzymes from the class of ligases [51]. One of the few examples for the biotechnological application of an enzyme from the group of ligases was described in 1998 by Aresta et al. [135]. They reported about a cheap, fast, and easy method for the phosphorylation of phenol at room temperature and subatmospheric pressure of CO₂: catalyzed by phenyl phosphate carboxylase, the synthesis of 4-OH benzoic acid from phenol and CO₂ with 100% selectivity was achieved (Figure 33.30).

Ligases also play an important role in the field of genetic engineering: DNA ligases (6.5.1.1) have become an indispensable tool for generating recombinant DNA sequences in modern molecular biology. By the development of a variety of nucleic acid-based detection systems for genetic disorders as well as for bacterial, viral, and other pathogens, ligases have been applied in a number of DNA amplification methods including polymerase chain reaction, self-sustained sequence replication, Q-beta replicase, and ligase chain reaction.

The discovery of DNA ligases and the biochemical studies of the ligase reaction by Lehman et al. were the first reports about host and bacteriophage-induced DNA ligases from eubacteria [136]. Subsequently, related enzymes from a wide range of organisms have been identified and studied. Today it is known that DNA ligases represent a large family of evolutionarily related proteins that play an important role in many essential reactions within the living cell including replication, recombination, and repair of DNA in all three kingdoms

TABLE 33.15
Subclasses of the Ligases

EC 6 Ligases	
EC 6.1	Forming carbon–oxygen bonds
EC 6.2	Forming carbon–sulfur bonds
EC 6.3	Forming carbon–nitrogen bonds
EC 6.4	Forming carbon–carbon bonds
EC 6.5	Forming phosphoric ester bonds
EC 6.6	Forming nitrogen–metal bonds

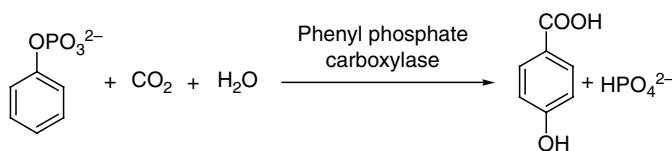


FIGURE 33.30 Synthesis of 4-OH benzoic acid from phenol and CO_2 by phenyl phosphate carboxylase. (From Aresta, M. et al., *Tetrahedron*, 54, 8841–8846, 1998.)

of life. They catalyze the formation of phosphodiester bonds at single-stranded breaks (nicks) between adjacent 3'-hydroxyl and 5'-phosphate termini in double-stranded DNA by using either ATP or NAD^+ as a cofactor [136,137].

As the presence of NAD^+ -dependent DNA ligases is restricted to eubacteria, this fact makes them an attractive target for novel antibiotics. For example, bacterial NAD^+ -synthetase (EC 6.3.5.1) catalyzes the last step in both the *de novo* biosynthetic and salvage pathways for NAD^+ and thus plays an essential role in the life cycle of bacteria. In order to find novel inhibitors for this enzyme for future antibacterial drug development, an enzymatic assay was developed by Yang et al. for the purpose of screening compounds for enzyme inhibition [138]. To improve the solubility of the compounds screened, the water-miscible organic solvent dimethyl sulfoxide (DMSO) was added to the assay buffer as cosolvent. Although no effects could be observed on behalf of the enzyme activity, concentrations of 2.5% (v/v) DMSO led to changes in the stability of the dimer and its unfolding mechanism.

Although bacterial NAD^+ -dependent DNA ligases have been studied for more than 30 years, surprisingly few genetic and biochemical details are known about their regulation. In order to find new and interesting targets for future antibacterial drug development further investigations will be needed.

To summarize, ligases represent a class of enzymes found ubiquitously in nature. Due to their involvement in numerous essential reactions within the living cell and their cofactor dependency, the catalytic activity of ligases is more or less restricted to aqueous media, though some might act in the presence of small concentrations of cosolvents as well. While the subclass of DNA-ligases has already found numerous applications in molecular biology, the discovery of other ligases, e.g., acetyl-coenzyme A carboxylase (ACC) (EC 6.4.1.2), will be useful for the investigation of future potential targets for drug discovery. ACC plays a crucial role in fatty acid metabolism in most living organisms. It catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA and represents an attractive target for the therapeutic intervention in the control of obesity and the treatment of metabolic syndrome [139].

REFERENCES

1. Zaks, A. and Klivanov, A.M., Enzyme-catalyzed processes in organic solvents, *Proc. Natl. Acad. Sci. USA.*, 82, 3192–3196, 1985.
2. Kilbanov, A.M., Enzymes that work in organic solvents, *Chemtech*, 16, 354–359, 1986.
3. Klivanov, A.M., Why are enzymes less active in organic solvents than in water? *Trends Biotechnol.*, 15, 97–101, 1997.
4. Carrea, G., Biocatalysis in water–organic solvent 2-phase systems, *Trends Biotechnol.*, 2, 102–106, 1984.
5. Halling, P.J., Biocatalysis in multiphase reaction mixtures containing organic liquids, *Biotechnol. Adv.*, 5, 47–84, 1987.
6. Shimizu, S. and Yamada, H., Stereospecific reduction of 3-keto acid-esters by a novel aldehyde reductase of *Sporobolomyces salmonicolor* in a water organic solvent 2-phasic system, *Ann. NY Acad. Sci.*, 613, 628–632, 1990.

7. Halling, P.J., Thermodynamic predictions for biocatalysis in nonconventional media—theory, tests, and recommendations for experimental design and analysis, *Enzyme Microb. Technol.*, 16, 178–206, 1994.
8. Heinemann, M. et al., Experimental and theoretical analysis of phase equilibria in a two-phase system used for biocatalytic esterifications, *Biocatal. Biotrans.*, 21, 115–121, 2003.
9. Martinek, K. et al., Preparative enzymatic synthesis in biphasic aqueous–organic system, *Bioorganicheskaya Khimiya*, 3, 696–702, 1977.
10. Laane, C., Tramper, J., and Lilly, M.D., *Biocatalysis in Organic Media*, Elsevier, Amsterdam, 1987.
11. van Deurzen, M.P.J. et al., Chloroperoxidase-catalyzed oxidations in *t*-butyl alcohol/water mixtures, *J. Mol. Catal. A Chemical*, 117, 329–337, 1997.
12. Patel, R., *Stereoselective Biocatalysis*, Marcel Dekker, New York, 2000, p. 799.
13. Cull, S.G. et al., Room-temperature ionic liquids as replacements for organic solvents in multiphase bioprocess operations, *Biotechnol. Bioeng.*, 69, 227–233, 2000.
14. Erbdinger, M., Mesiano, A.J., and Russell, A.J., Enzymatic catalysis of formation of Z-aspartame in ionic liquid—an alternative to enzymatic catalysis in organic solvents, *Biotechnol. Prog.*, 16, 1129–1131, 2000.
15. Kragl, U., Eckstein, M., and Kaftzik, N., Enzyme catalysis in ionic liquids, *Curr. Opin. Biotechnol.*, 13, 565–571, 2002.
16. van Rantwijk, F., Lau, R.M., and Sheldon, R.A., Biocatalytic transformations in ionic liquids, *Trends Biotechnol.*, 21, 131–138, 2003.
17. Wong, C.-H. and Whitesides, G.M., *Enzymes in Synthetic Organic Chemistry*, Pergamon Press, Oxford, 1994, pp. 12, 370.
18. Hummel, W., Reduction of acetophenone to *R*(+)-phenylethanol by a new alcohol dehydrogenase from *Lactobacillus kefir*, *Appl. Microbiol. Biotechnol.*, 34, 15–19, 1990.
19. Howarth, J., James, P., and Dai, J.F., Immobilized baker's yeast reduction of ketones in an ionic liquid, [bmim]PF₆ and water mix, *Tetrahedron Lett.*, 42, 7517–7519, 2001.
20. Kragl, U., Eckstein, M., and Kaftzik, N., Enzyme catalysis in ionic liquids, *Curr. Opin. Biotechnol.*, 13, 565–571, 2002.
21. Nakamura, K. et al., Recent developments in asymmetric reduction of ketones with biocatalysts, *Tetrahedron Asymmetry*, 14, 2659–2681, 2003.
22. Matsue, S. and Miyawaki, O., Influence of water activity and aqueous solvent ordering on enzyme kinetics of alcohol dehydrogenase, lysozyme, and beta-galactosidase, *Enzyme Microb. Technol.*, 26, 342–347, 2000.
23. Liao, M.H. and Chen, D.H., Characteristics of magnetic nanoparticles-bound YADH in water/AOT/isooctane microemulsions, *J. Mol. Catal. B Enzym.*, 18, 81–87, 2002.
24. Hirakawa, H. et al., Regioselective reduction of a steroid in a reversed micellar system with enzymatic NADH-regeneration, *Biochem. Eng. J.*, 16, 35–40, 2003.
25. Chen, D.H. and Liao, M.H., Effects of mixed reverse micellar structure on stability and activity of yeast alcohol dehydrogenase, *J. Mol. Catal. B Enzym.*, 18, 155–162, 2002.
26. Riebel, B., Biochemische und molekularbiologische Charakterisierung neuer mikrobieller NAD(P)-abhängiger Alkoholdehydrogenasen, University Düsseldorf, Düsseldorf, 1996.
27. Villela Filho, M. et al., Is the log *P* a convenient criterion to guide the choice of solvents for biphasic enzymatic reactions? *Angew. Chem.* 115, 3101–3104, 2003.
28. Ferloni, C. et al., Enzymatische Gasphasenkatalyse zur Produktion chiraler Substanzen, *Biokatalyse* (Transkript Sonderheft), 105–108, 2003.
29. Eckstein, M., Daubmann, T., and Kragl, U., Recent development in NAD(P)H regeneration for enzymatic reductions in one- and two-phase systems, *Biocatal. Biotrans.*, 22, 89–96, 2004.
30. Schumacher, J., Eckstein, M., and Kragl, U., Influence of water-miscible organic solvents on kinetics and enantioselectivity of the (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis*, *Biotechnol. J.*, 1, 574–581, 2006.
31. Eckstein, M. et al., Use of an ionic liquid in a two-phase system to improve an alcohol dehydrogenase catalysed reduction, *Chem. Comm.*, 1084–1085, 2004.
32. Kroutil, W. et al., Recent advances in the biocatalytic reduction of ketones and oxidation of sec-alcohols, *Curr. Opin. Chem. Biol.*, 8, 120–126, 2004.

33. Eckstein, M. et al., Maximise your equilibrium conversion in biphasic catalysed reactions: How to obtain reliable data for equilibrium constants? *Adv. Syn. Catal.*, in press.
34. Kragl, U. et al., Repetitive batch as an efficient method for preparative scale enzymic synthesis of 5-azido-neuroaminic acid and 15*N*-L-glutamic acid, *Tetrahedron Asymmetry*, 4, 1193–1202, 1993.
35. Adlercreutz, P., Cofactor regeneration in biocatalysis in organic media, *Biocatal. Biotrans.*, 14, 1–30, 1996.
36. De Temino, D.M., Hartmeier, W., and Ansorge-Schumacher, M.B., Entrapment of the alcohol dehydrogenase from *Lactobacillus kefir* in polyvinyl alcohol for the synthesis of chiral hydrophobic alcohols in organic solvents, *Enzyme Microb. Technol.*, 36, 3–9, 2005.
37. Mateo, C. et al., A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates, *Biotechnol. Bioeng.*, 86, 273–276, 2004.
38. Khmelnitsky, Y.L. et al., Denaturation capacity—a new quantitative criterion for selection of organic-solvents as reaction media in biocatalysis, *Eur. J. Biochem.* 198, 31–41, 1991.
39. Tanaka, T. et al., Enzymatic oxidative polymerization of 4-chloroguaiacol by laccase, *J. Chem. Eng. Jap.*, 36, 1101–1106, 2003.
40. Pilz, R. et al., Laccase-catalysed synthesis of coupling products of phenolic substrates in different reactors, *Appl. Microbiol. Biotechnol.*, 60, 708–712, 2003.
41. Nicotra, S. et al., Laccase-mediated oxidation of the steroid hormone 17 beta-estradiol in organic solvents, *Tetrahedron Asymmetry*, 15, 2927–2931, 2004.
42. Lugaro, G., Carrea, G., and Cremonesi, P., The oxidation of steroid hormones by fungal laccase in emulsion of water and organic solvents, *Arch. Biochem. Biophys.*, 159, 1–6, 1973.
43. Schmid, A. et al., Integrated biocatalytic synthesis on gramm scale: the highly enantioselective preparation of chiral oxiranes with styrene monooxygenase, *Adv. Synth. Catal.*, 343, 732–737, 2001.
44. Hofstetter, K. et al., Coupling of biocatalytic asymmetric epoxidation with NADH regeneration in organic–aqueous emulsions, *Angew. Chem. Int. Ed.*, 43, 2163–2166, 2004.
45. Zambianchi, F. et al., Use of isolated cyclohexanone monooxygenase from recombinant *Escherichia coli* as a biocatalyst for Baeyer–Villiger and sulfide oxidations, *Biotechnol. Bioeng.*, 78, 489–496, 2002.
46. Hilker, I. et al., Microbiological transformations. 57. Facile and efficient resin-based *in situ* SFPR preparative-scale synthesis of an enantiopure “unexpected” lactone regioisomer via a Baeyer–Villiger oxidation process, *Org. Lett.*, 6, 1955–1958, 2004.
47. Anderson, B.A. et al., Application of a practical biocatalytic reduction to an enantioselective synthesis of the 5H-2,3-benzodiazepine Ly300164, *J. Am. Chem. Soc.*, 117, 12358–12359, 1995.
48. Vicenzi, J.T. et al., Large-scale stereoselective enzymatic ketone reduction with *in situ* product removal via polymeric adsorbent resins, *Enzyme Microb. Technol.*, 20, 494–499, 1997.
49. Michizoe, J. et al., Functionalization of the cytochrome P450cam monooxygenase system in the cell-like aqueous compartments of water-in-oil emulsions, *J. Biosci. Bioeng.*, 99, 12–17, 2005.
50. <http://www.brenda.uni-koeln.de/>.
51. Faber, K., *Biotransformations in Organic Chemistry*, 5th ed., Springer, Berlin, 2004.
52. Wu, J.Y. and Yang, J.T., Effects of salts and organic solvents on activity of citrate synthase, *J. Biol. Chem.*, 245, 3561, 1970.
53. Singh, T.J. and Wang, J.H., Stimulation of glycogen-phosphorylase kinase from rabbit skeletal-muscle by organic-solvents, *J. Biol. Chem.*, 254, 8466–8472, 1979.
54. Rajala, R.V.S. and Sharma, R.K., Myristoyl CoA-Protein *N*-myristoyltransferase—subcellular-localization, activation and kinetic-behavior in the presence of organic-solvents, *Biochem. Biophys. Res. Commun.*, 208, 617–623, 1995.
55. Singer, S.J., The properties of proteins in nonaqueous solvents, *Adv. Prot. Chem.*, 17, 1–68, 1962.
56. Shin, J.S. and Kim, B.G., Kinetic resolution of alpha-methylbenzylamine with omega-transaminase screened from soil microorganisms: application of a biphasic system to overcome product inhibition, *Biotechnol. Bioeng.*, 55, 348–358, 1997.
57. Bornscheuer, U.T. and Kazlauskas, R.J., *Hydrolases in Organic Synthesis. Regio- and Stereoselective Biotransformations*, Wiley-VCH, Weinheim, Germany, 1999.

58. Verger, R., 'Interfacial activation' of lipases: facts and artifacts, *Trends Biotechnol.*, 15, 32–38, 1997.
59. Schmid, R.D. and Verger, R., Lipases: interfacial enzymes with attractive applications, *Angew. Chem. Int. Ed.*, 37, 1609–1633, 1998.
60. Pottie, M., Vandereycken, J., and Vandewalle, M., Enzymatic enantioselective hydrolysis of 2,2-dimethyl-1,3-dioxolane-4-carboxylic esters, *Tetrahedron Lett.*, 30, 5319–5322, 1989.
61. Dumortier, L., Vandereycken, J., and Vandewalle, M., The synthesis of chiral isopropylidene derivatives of 1,2,3-cyclohexanetriols by enzymatic differentiation, *Tetrahedron Lett.*, 30, 3201–3204, 1989.
62. Xie, Z.F. et al., An insight into the enantioselective hydrolyses of cyclic acetates catalyzed by *Pseudomonas fluorescens* lipase, *J. Chem. Soc. Chem. Commun.*, 966–977, 1988.
63. Seemayer, R. and Schneider, M.P., Enzymatic preparation of tetrahydrofuran derivatives of high optical purity, *J. Chem. Soc. Perkin 1*, 2359–2360, 1990.
64. Laumen, K. and Schneider, M.P., A highly selective ester hydrolase from *Pseudomonas* sp. for the enzymatic preparation of enantiomerically pure secondary alcohols—chiral auxiliaries in organic-synthesis, *J. Chem. Soc. Chem. Commun.*, 598–600, 1988.
65. Kalaritis, P. et al., Kinetic resolution of 2-substituted esters catalyzed by a lipase ex *Pseudomonas fluorescens*, *J. Org. Chem.*, 55, 812–815, 1990.
66. Hughes, D.L. et al., Lipase-catalyzed asymmetric hydrolysis of esters having remote chiral prochiral centers, *J. Org. Chem.*, 55, 6252–6259, 1990.
67. Halling, P. and Kvittingen, L., Why did biocatalysis in organic media not take off in the 1930s? *Trends Biotechnol.*, 17, 343–344, 1999.
68. Sym, E.A., Action of esterase in the presence of organic solvents, *Biochem. J.*, 30, 609–617, 1936.
69. Sperry, W.M. and Brand, F.C., A study of cholesterol esterase in liver and brain, *J. Biol. Chem.*, 137, 377–387, 1941.
70. Cambou, B. and Klibanov, A.M., Unusual catalytic properties of usual enzymes, *Ann. NY Acad. Sci.*, 434, 219–223, 1984.
71. Mori, K. et al., Pheromone synthesis. 101. Synthesis and bioactivity of optically active forms of 1-methyl-2-cyclohexen-1-ol, an aggregation pheromone *Dendroctonus-pseudotsugae*, *Tetrahedron*, 43, 2249–2254, 1987.
72. Johnston, B.D. et al., A convenient synthesis of both enantiomers of seudenol and their conversion to 1-methyl-2-cyclohexen-1-ol (Mcol), *Tetrahedron Asymmetry*, 2, 377–380, 1991.
73. Hartmeier, W., *Immobilized Biocatalysts: An Introduction.*, Springer, Berlin, 1988.
74. Brandenburg, H., Methods for linking enzymes to insoluble carriers, *Angew. Chem. Int. Ed.*, 67, 244–246, 1955.
75. Reetz, M.T. et al., Characterization of hydrophobic sol–gel materials containing entrapped lipases, *J. Sol–Gel Sci. Technol.*, 7, 35–43, 1996.
76. Buthe, A. et al., Generation of lipase-containing static emulsions in silicone spheres for synthesis in organic media, *J. Mol. Catal. B Enzym.*, 35, 93–99, 2005.
77. Goto, M., Hatanaka, C., and Goto, M., Immobilization of surfactant–lipase complexes and their high heat resistance in organic media, *Biochem. Eng. J.*, 24, 91–94, 2005.
78. Palomo, J.M. et al., Modulation of the enantioselectivity of lipases via controlled immobilization and medium engineering: hydrolytic resolution of mandelic acid esters, *Enzyme Microb. Technol.*, 31, 775–783, 2002.
79. Hilal, N., Nigmatullin, R., and Alpatova, A., Immobilization of cross-linked lipase aggregates within microporous polymeric membranes, *J. Membrane Sci.*, 238, 131–141, 2004.
80. Wasserscheid, P. and Keim, W., Ionic liquids: new “solutions” for transition metal catalysis, *Angew. Chem. Int. Ed.*, 39, 3773–3789, 2000.
81. Welton, T., Room-temperature ionic liquids: solvents for synthesis and catalysis, *Chem. Rev.*, 99, 2071–2083, 1999.
82. Seddon, K.R., Ionic liquids for clean technology, *J. Chem. Technol. Biotechnol.*, 68, 351–356, 1997.
83. Lau, R.M. et al., Lipase-catalyzed reactions in ionic liquids, *Org. Lett.*, 2, 4189–4191, 2000.
84. Schofer, S.H. et al., Enzyme catalysis in ionic liquids: lipase catalysed kinetic resolution of 1-phenylethanol with improved enantioselectivity, *Chem. Commun.*, 425–426, 2001.

85. Itoh, T., Akasaki, E., and Nishimura, Y., Efficient lipase-catalyzed enantioselective acylation under reduced pressure conditions in an ionic liquid solvent system, *Chem. Lett.*, 154–155, 2002.
86. Park, S. and Kazlauskas, R.J., Improved preparation and use of room-temperature ionic liquids in lipase-catalyzed enantio- and regioselective acylations, *J. Org. Chem.*, 66, 8395–8401, 2001.
87. Skagerlind, P., Jansson, M., and Hult, K., Surfactant interference on lipase-catalyzed reactions in microemulsions, *J. Chem. Technol. Biotechnol.*, 54, 277–282, 1992.
88. Bello, M., Thomas, D., and Legoy, M.D., Interesterification and synthesis by *Candida cylindracea* lipase in microemulsions, *Biochem. Biophys. Res. Commun.*, 146, 361–367, 1987.
89. Hedstrom, G., Backlund, M., and Slotte, J.P., Enantioselective synthesis of ibuprofen esters in AOT isooctane microemulsions by *Candida cylindracea* lipase, *Biotechnol. Bioeng.*, 42, 618–624, 1993.
90. Krieger, N. et al., Non-aqueous biocatalysis in heterogeneous solvent systems, *Food Technol. Biotechnol.*, 42, 279–286, 2004.
91. Nakamura, K. et al., Lipase activity and stability in supercritical carbon-dioxide, *Chem. Eng. Commun.*, 45, 207–212, 1986.
92. Reetz, M.T. et al., Continuous flow enzymatic kinetic resolution and enantiomer separation using ionic liquid/supercritical carbon dioxide media, *Adv. Synth. Catal.*, 345, 1221–1228, 2003.
93. Lozano, P. et al., Continuous green biocatalytic processes using ionic liquids and supercritical carbon dioxide, *Chem. Commun.*, 692–693, 2002.
94. Reetz, M.T. et al., Biocatalysis in ionic liquids: batchwise and continuous flow processes using supercritical carbon dioxide as the mobile phase, *Chem. Commun.*, 992–993, 2002.
95. Ohno, M. and Otsuka, M., Chiral synthons by ester hydrolysis catalysed by pig liver esterase, *Org. React.*, 37, 1–55, 1990.
96. Faber, K., Ottolina, G., and Riva, S., Selectivity-enhancement of hydrolase reactions, *Biocatalysis*, 8, 91–132, 1993.
97. Ruppert, S. and Gais, H.J., Activity enhancement of pig liver esterase in organic solvents by colyophilization with methoxypolyethylene glycol: kinetic resolution of alcohols, *Tetrahedron Asymmetry*, 8, 3657–3664, 1997.
98. Mohr, P. et al., A study of stereoselective hydrolysis of symmetrical diesters with pig-liver esterase, *Helv. Chim. Acta*, 66, 2501–2511, 1983.
99. Schneider, M. et al., Hydrolytic enzymes in organic-synthesis. 3. Enzymatic syntheses of chiral building-blocks from prochiral meso-substrates preparation of methyl(hydrogen)-1,2-cycloalkanedicarboxylates, *Angew. Chem. Int. Ed. Engl.*, 23, 67–68, 1984.
100. Anwar, A. and Saleemuddin, M., Alkaline proteases: a review, *Bioresour. Technol.*, 64, 175–183, 1998.
101. Gupta, R., Beg, Q.K., and Lorenz, P., Bacterial alkaline proteases: molecular approaches and industrial applications, *Appl. Microbiol. Biotechnol.*, 59, 15–32, 2002.
102. Isowa, Y. et al., Thermolysin-catalyzed condensation-reactions of N-substituted aspartic and glutamic acids with phenylalanine alkyl esters, *Tetrahedron Lett.*, 28, 2611–2612, 1979.
103. Oyama, K., *Industrial Production of Aspartame*, in: *Chirality in Industry*, Wiley, Chichester, 1992, pp. 237–247.
104. Wandrey, C., Liese, A., and Kihumbu, D., Industrial biocatalysis: past, present, and future, *Org. Proc. Res. Dev.*, 4, 286–290, 2000.
105. Glieder, A. et al., Comprehensive step-by-step engineering of an (*R*)-hydroxynitrile lyase for large-scale asymmetric synthesis, *Angew. Chem. Int. Ed.*, 42, 4815–4818, 2003.
106. Nagasawa, T., Shimizu, H., and Yamada, H., The superiority of the 3rd-generation catalyst, *Rhodococcus rhodochrous* J1 nitrile hydratase, for industrial-production of acrylamide, *Appl. Microbiol. Biotechnol.*, 40, 189–195, 1993.
107. Watanabe, I., Satoh, Y., and Enomoto, K., Screening, isolation and taxonomical properties of microorganisms having acrylonitrile-hydrating activity, *Agric. Biol. Chem.*, 51, 3193–3199, 1987.
108. Watanabe, I. et al., Optimal conditions for cultivation of *Rhodococcus* sp. N-774 and for conversion of acrylonitrile to acrylamide by resting cells, *Agric. Biol. Chem.*, 51, 3201–3206, 1987.
109. Martinkova, L. and Mylerova, V., Synthetic applications of nitrile-converting enzymes, *Curr. Org. Chem.*, 7, 1279–1295, 2003.
110. Layh, N. and Willetts, A., Enzymatic nitrile hydrolysis in low water systems, *Biotechnol. Lett.*, 20, 329–331, 1998.

111. Bauer, M., Griengl, H., and Steiner, W., Kinetic studies on the enzyme (S)-hydroxynitrile lyase from *Hevea brasiliensis* using initial rate methods and progress curve analysis, *Biotechnol. Bioeng.*, 62, 20–29, 1999.
112. Reisinger, C. et al., Enzymatic hydrolysis of cyanohydrins with recombinant nitrile hydratase and amidase from *Rhodococcus erythropolis*, *Biotechnol. Lett.*, 26, 1675–1680, 2004.
113. Sheldon, R.A., Green solvents for sustainable organic synthesis: state of the art, *Green Chem.*, 7, 267–278, 2005.
114. Fechter, M.H. and Griengl, H., Hydroxynitrile lyases: biological sources and application as biocatalysts, *Food Technol. Biotechnol.*, 42, 287–294, 2004.
115. Rosenthaler, L., Durch Enzyme bewirkte asymmetrische Synthesen, *Biochemische Zeitschrift: Beiträge zur chem. Physiologie u. Pathologie*, 14, 238–253, 1908.
116. Hickel, A., Hasslacher, M., and Griengl, H., Hydroxynitrile lyases: functions and properties, *Physiol. Plant.*, 98, 891–898, 1996.
117. Buhler, H., Bayer, A., and Effenberger, F., A convenient synthesis of optically active 5,5-disubstituted 4-amino- and 4-hydroxy-2(5H)-furanones from (S)-ketone cyanohydrins, *Chem. Eur. J.*, 6, 2564–2571, 2000.
118. Buhler, H. et al., Substrate specificity of mutants of the hydroxynitrile lyase from *Manihot esculenta*, *ChemBioChem*, 4, 211–216, 2003.
119. Buhler, H., Miehlisch, B., and Effenberger, F., Inversion of stereoselectivity by applying mutants of the hydroxynitrile lyase from *Manihot esculenta*, *ChemBioChem*, 6, 711–717, 2005.
120. Buhler, H., Optimierung der (S)-Hydroxynitril Lyase aus *Manihot esculenta* durch gezielte Mutationen—Anwendungen von optisch aktiven Cyanhydrinen in der Synthese, University Stuttgart, Stuttgart, Germany, 2000.
121. Griengl, H. et al., Enzyme catalysed formation of (S)-cyanohydrins derived from aldehydes and ketones in a biphasic solvent system, *Tetrahedron*, 54, 14477–14486, 1998.
122. Lou, W.Y., Xu, R., and Zong, M.H., Hydroxynitrile lyase catalysis in ionic liquid-containing systems, *Biotechnol. Lett.*, 27, 1387–1390, 2005.
123. Hickel, A., Radke, C.J., and Blanch, H.W., Hydroxynitrile lyase at the diisopropyl ether/water interface: evidence for interfacial enzyme activity, *Biotechnol. Bioeng.*, 65, 425–436, 1999.
124. Trummler, K. and Wajant, H., Molecular cloning of acetone cyanohydrin lyase from flax (*Linum usitatissimum*): definition of a novel class of hydroxynitrile lyases, *J. Biol. Chem.*, 272, 4770–4774, 1997.
125. Yan, G.H. et al., A single residual replacement improves the folding and stability of recombinant cassava hydroxynitrile lyase in *E. coli*, *Biotechnol. Lett.*, 25, 1041–1047, 2003.
126. Hao, J.J. and Berry, A., A thermostable variant of fructose biphosphate aldolase constructed by directed evolution also shows increased stability in organic solvents, *Prot. Eng. Des. Sel.*, 17, 689–697, 2004.
127. Choi, I.G. et al., Overproduction, purification, and characterization of heat-stable aldolase from *Methanococcus jannaschii*, a hyperthermophilic archaea, *J. Biochem. Mol. Biol.*, 31, 130–134, 1998.
128. Yagasaki, M. and Ozaki, A., Industrial biotransformations for the production of D-amino acids, *J. Mol. Catal. B Enzym.*, 4, 1–11, 1998.
129. Gallo, K.A. and Knowles, J.R., Purification, cloning, and cofactor independence of glutamate racemase from *Lactobacillus*, *Biochemistry*, 32, 3981–3990, 1993.
130. Lim, Y.H. et al., A new amino-acid racemase with threonine alpha-epimerase activity from *Pseudomonas putida*: purification and characterization, *J. Bacteriol.*, 175, 4213–4217, 1993.
131. Asano, Y. and Yamaguchi, S., Discovery of amino acid amides as new substrates for alpha-amino-epsilon-caprolactam racemase from *Achromobacter obae*, *J. Mol. Catal. B Enzym.*, 36, 22–29, 2005.
132. Felfer, U. et al., Substrate spectrum of mandelate racemase—Part 2. (Hetero)-aryl-substituted mandelate derivatives and modulation of activity, *J. Mol. Catal. B Enzym.*, 15, 213–222, 2001.
133. Strauss, U.T. and Faber, K., Deracemization of (+/-)-mandelic acid using a lipase-mandelate racemase two-enzyme system, *Tetrahedron Asymmetry*, 10, 4079–4081, 1999.
134. Kaftzik, N. et al., Mandelate racemase activity in ionic liquids: scopes and limitations, *J. Mol. Catal. A Chem.*, 214, 107–112, 2004.

135. Aresta, M. et al., Enzymatic synthesis of 4-OH-benzoic acid from phenol and CO₂: the first example of a biotechnological application of a carboxylase enzyme, *Tetrahedron*, 54, 8841–8846, 1998.
136. Lehman, I.R., DNA ligase: structure, mechanism, and function, *Science*, 186, 790–797, 1974.
137. Lindahl, T. and Barnes, D.E., Mammalian DNA ligases, *Annu. Rev. Biochem.*, 61, 251–281, 1992.
138. Yang, Z.R.W. et al., Dimethyl sulfoxide at 2.5% (v/v) alters the structural cooperativity and unfolding mechanism of dimeric bacterial NAD(+) synthetase, *Prot. Sci.*, 13, 830–841, 2004.
139. Tong, L., Acetyl-coenzyme A carboxylase: crucial metabolic enzyme and attractive target for drug discovery, *Cell. Mol. Life Sci.*, 62, 1784–1803, 2005.
140. Jaeger, K.E. and Reetz, M.T., Microbial lipases form versatile tools for biotechnology, *Trends Biotechnol.*, 16, 396–403, 1998.
141. Pandey, A. et al., The realm of microbial lipases in biotechnology, *Biotechnol. Appl. Biochem.*, 29, 119–131, 1999.
142. Farrell, R.L., Hata, K., and Wall, M.B., Solving pitch problems in pulp and paper processes by the use of enzymes or fungi, *Adv. Biochem. Eng. Biotechnol.*, 57, 197–212, 1997.
143. Parmar, V.S. et al., Chiral discrimination by hydrolytic enzymes in the synthesis of optically pure materials, *Proc. Ind. Acad. Sci. Chem. Sci.*, 108, 575–583, 1996.
144. Rehm, H.J., Reed, G., and Stadler, P., *Biotransformations I—Water, Organic Solvents and Other Reaction Media*, VCH, Weinheim, Germany, 1998, 8a,
145. Shoemaker, M. et al., The lipodygenase sensor, a new approach in essential fatty acid determination in foods, *Biosens. Bioelectron.*, 12, 1089–1099, 1997.
146. Godfrey, T. and West, S.H., *Industrial Enzymology: The Application of Enzymes in Industry*, Stockton, New York, 1996,
147. Izumi, T. et al., Enzymatic transesterification of 3,7-dimethyl-4,7-octadien-1-ol using lipases, *J. Chem. Technol. Biotechnol.*, 68, 57–64, 1997.
148. Salleh, A.B. et al., Extracellular and intracellular lipases from a thermophilic *Rhizopus oryzae* and factors affecting their production, *Can. J. Microbiol.*, 39, 978–981, 1993.
149. Kaneki, H. and Tanaka, M., Activation of *Rhizopus delemar* lipase-catalyzed hydrolysis of tripropionin by DDT and aldrin: tightly bound pesticide-lipase complexes, *Eisei Kagaku—Jap. J. Toxicol. Environ. Health*, 32, 1–12, 1986.
150. Boland, W., Frossl, C., and Lorenz, M., Esterolytic and lipolytic enzymes in organic-synthesis, *Synth. Stutt.*, 1049–1072, 1991.
151. Otera, J., Transesterification, *Chem. Rev.*, 93, 1449–1470, 1993.
152. Theil, F., Lipase-supported synthesis of biologically active compounds, *Chem. Rev.*, 95, 2203–2227, 1995.
153. West, S.I., Enzymes in the food-processing industry, *Chem. Br.*, 24, 1220–1222, 1988.
154. Gotor, V., Non-conventional hydrolase chemistry: amide and carbamate bond formation catalyzed by lipases, *Bioorg. Med. Chem.*, 7, 2189–2197, 1999.
155. Fernandes, M.L.M. et al., Hydrolysis and synthesis reactions catalysed by *Thermomyces lanuginosa* lipase in the AOT/isooctane reversed micellar system, *J. Mol. Catal. B Enzym.*, 30, 43–49, 2004.
156. Falcone, R.D. et al., Effect of the addition of a nonaqueous polar solvent (Glycerol) on enzymatic catalysis in reverse micelles. Hydrolysis of 2-naphthyl acetate by alpha-chymotrypsin, *Langmuir*, 20, 5732–5737, 2004.
157. Naoe, K. et al., Solvent condition in triolein hydrolysis by *Rhizopus delemar* lipase using an AOT reverse micellar system, *Biochem. Eng. J.*, 18, 49–55, 2004.
158. Altreuter, D.H., Dordick, J.S., and Clark, D.S., Optimization of ion-paired lipase for non-aqueous media: acylation of doxorubicin based on surface models of fatty acid esterification, *Enzyme Microb. Technol.*, 31, 10–19, 2002.
159. Zhou, G.W. et al., Kinetic studies of lipase-catalyzed esterification in water-in-oil microemulsions and the catalytic behavior of immobilized lipase in MBGs, *Colloids Surf. A Physicochem. Eng. Aspects*, 194, 41–47, 2001.
160. Knez, Z. and Habulin, M., Compressed gases as alternative enzymatic-reaction solvents: a short review, *J. Supercrit. Fluids*, 23, 29–42, 2002.

161. Lozano, P. et al., Synthesis of glycidyl esters catalyzed by lipases in ionic liquids and supercritical carbon dioxide, *J. Mol. Catal. A Chem.*, 214, 113–119, 2004.
162. Madras, G., Kolluru, C., and Kumar, R., Synthesis of biodiesel in supercritical fluids, *Fuel*, 83, 2029–2033, 2004.
163. Sovova, H. and Zarevucka, M., Lipase-catalysed hydrolysis of blackcurrant oil in supercritical carbon dioxide, *Chem. Eng. Sci.*, 58, 2339–2350, 2003.
164. Lozano, P. et al., Membrane reactor with immobilized *Candida antarctica* lipase B for ester synthesis in supercritical carbon dioxide, *J. Supercrit. Fluids*, 29, 121–128, 2004.
165. Moorlag, H. et al., Pig-liver esterase catalyzed hydrolyzes of racemic alpha-substituted alpha-hydroxy esters, *J. Org. Chem.*, 55, 5878–5881, 1990.
166. Sicsic, S., Leroy, J., and Wakselman, C., Geometric selectivity of pig-liver esterase and its application to the separation of fluorinated bicyclic esters, *Synth. Stutt.*, 155–156, 1987.
167. Papageorgiou, C. and Benezra, C., Use of enzymatic-hydrolysis of dimethyl malates for a short synthesis of tulipalin-b and of its enantiomer, *J. Org. Chem.*, 50, 1144–1145, 1985.
168. Pfründer Weuster-Botz, H., et al., Effiziente Ganzzell—Biotransformation im zweiphasigen System ionische Flüssigkeit Wasser, *Angew. Chem.*, 116, 4629–4631, 2004.

34 Biocatalytic Concepts for the Synthesis of Optically Active Amines

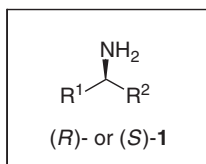
Stefan Buchholz and Harald Gröger

CONTENTS

34.1	Introduction	829
34.2	Kinetic Resolution	830
34.2.1	Overview about Types of Enzymatic Resolutions.....	830
34.2.2	Hydrolases-Catalyzed Resolution of Racemic Amines through Acylation....	832
34.2.3	Penicillin Acylase-Catalyzed Resolution of Racemic Amines through Acylation	836
34.2.4	Lipase-Catalyzed Resolution of Racemic Amides through Hydrolysis.....	836
34.2.5	Amine Oxidase-Catalyzed Resolution of Racemic Amines through Oxidation.....	838
34.2.6	Transaminase-Catalyzed Resolution of Racemic Amines through Oxidation.....	838
34.3	Dynamic Kinetic Resolution and Deracemization.....	840
34.3.1	Lipase-Catalyzed Dynamic Kinetic Resolution of Racemic Amines	841
34.3.2	Amine Oxidase-Catalyzed Deracemization of Racemic Amines	842
34.4	Asymmetric Synthesis	843
34.4.1	Asymmetric Transamination	843
34.5	Summary	845
	References	845

34.1 INTRODUCTION

A look into the structure of many drugs and agrochemicals often reveals a chiral amine subunit in the molecular framework [1]. Thus, there is a wide interest from the chemical and pharmaceutical industries for these types of optically active amines and manufacturing processes thereof. Many natural products, comprising highly complicated structures with various stereogenic centers, often contain chiral amine moieties (e.g., alkaloids and derivatives). Consequently, the synthesis of chiral amine building blocks is a field of intensive research in organic chemistry, and is also still challenging with respect to enzymatic approaches. In spite of the fact that numerous chiral amine structures exist in nature, most of the enzymatic synthetic approaches had not been developed until the 1990s [2]. On the other hand, biocatalysis offers a multitude of potential solutions covering a wide range of concepts based on resolution and asymmetric synthesis. Nevertheless, the “best solution” does not



SCHEME 34.1

exist, and different concepts turn out to be the preferred solution depending on the case studied.

This chapter covers the state of the art in the field of developed enantioselective synthetic methods for optically active amines **1** (Scheme 34.1) by means of biocatalysis and focuses on those reactions that lead to a chiral amine functionality itself. Thus, preparation of multi-functional compounds that bear an amino group but whose asymmetric synthesis is based on the modification of functional groups other than the amino group and the synthesis of amino acids in general are not included in this chapter as these are reviewed elsewhere [3]. An additional focus here will be on those biocatalytic routes that have already been proven on preparative, sometimes even technical, scale.

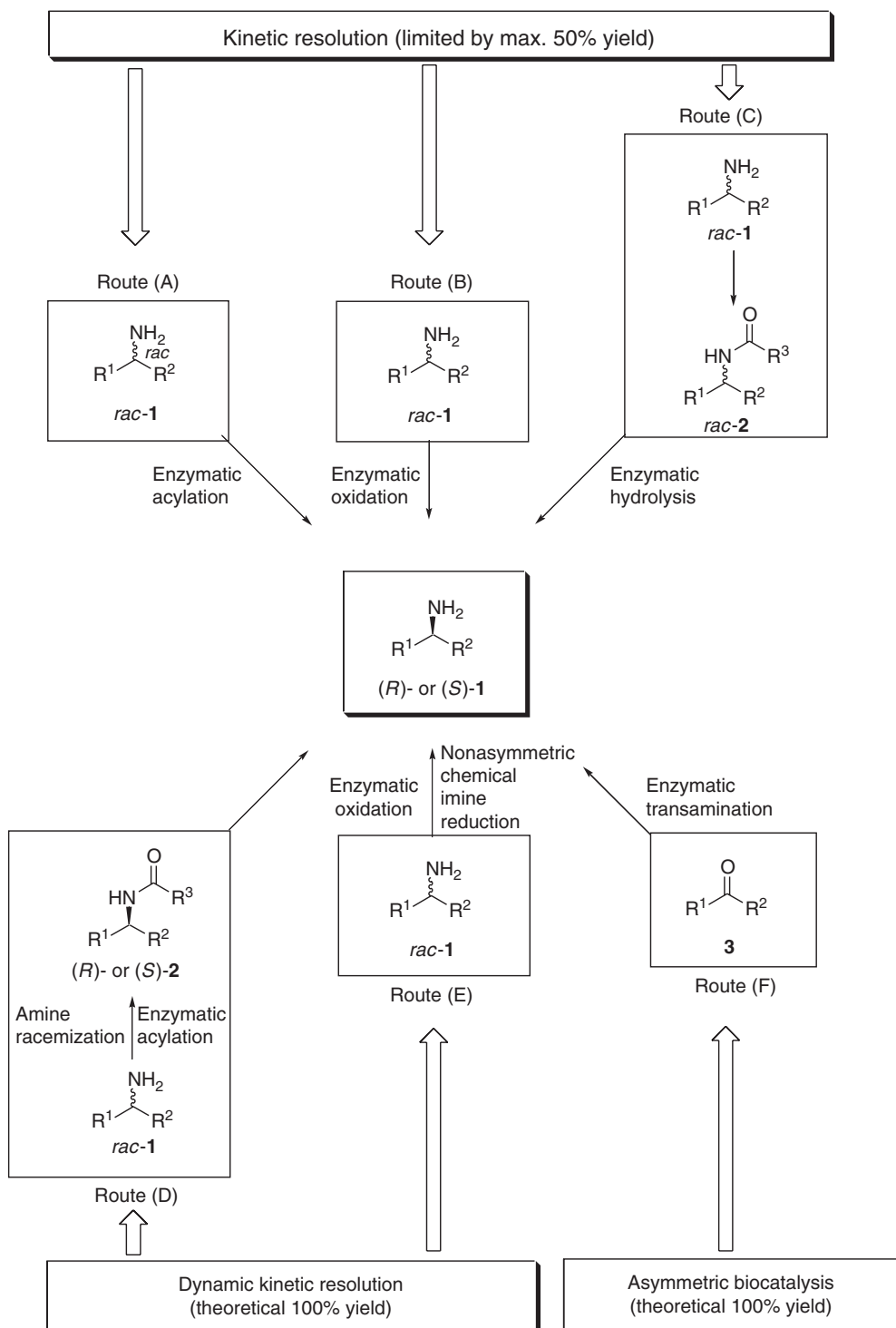
To start with the resolution concepts, a popular approach is the modification of the amine functionality in the presence of an enzyme capable of carrying out an acylation process. This approach, shown as Route (A) in Scheme 34.2, is in most cases based on the use of lipases in combination with an acyl donor, and the concept has been applied at industrial scale for quite some time already. Alternatively, amine oxidases can serve as suitable enzymes for the resolution of amines through enantioselective oxidation (Route (B)). Moreover, this kinetic resolution can be extended toward a deracemization process when combining it with a chemical *in situ* reduction of the imine intermediate formed during the oxidation step (Route (E)). Other types of enzymes suitable for conversions described in Route (B) are transaminases. In Route (C) a similar way—also following the concept of a resolution—that consists of a transformation of the racemic amine into an amide, followed by an enzymatic hydrolysis of the desired enantiomer under formation of the optically active amine is shown. With the exception of the extension toward a dynamic kinetic resolution process (known for lipase, see Route (D)) and deracemization (known for amine oxidase, see Route (E)), all resolutions are limited by a 50% conversion and require subsequent separation of the unwanted enantiomer and recycling.

A direct (theoretically) 100% approach toward optically active amines is possible when starting from prochiral compounds, namely ketones, as shown in Route (F). The required enzymes, which make this asymmetric catalytic process possible, are transaminases. This Route (F) has also already proven its feasibility on technical scale. Route (A) through Route (F) are subsequently described in more detail.

34.2 KINETIC RESOLUTION

34.2.1 OVERVIEW ABOUT TYPES OF ENZYMATIC RESOLUTIONS

Kinetic resolutions are a widely applied method for the synthesis of chiral building blocks. In spite of numerous examples for amino acids, carboxylic acids, alcohols, and many other types of molecules, it took surprisingly long until the first efficient resolutions for racemic amines were reported. Until now, kinetic resolutions according to Route (A) and Route (C) are known, based on the use of hydrolases, in particular lipases (for both Route (A) and Route



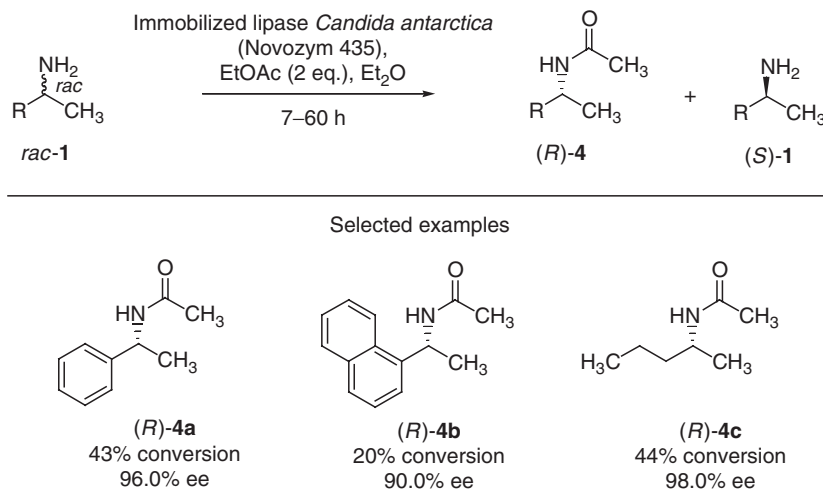
(C)), amine oxidases (Route (B) only), and transaminases (Route (B) only). Among these, the kinetic resolution with lipases according to Route (A), by means of an enantioselective acylation, plays an especially important role and has already become an industrially established method.

34.2.2 HYDROLASES-CATALYZED RESOLUTION OF RACEMIC AMINES THROUGH ACYLATION

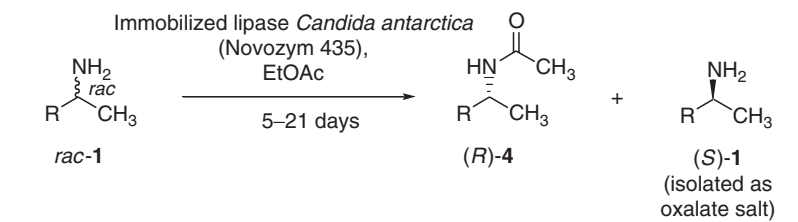
Although most of the hydrolases applied so far in the acylation of amines belong to the group of lipases, the first efficient example for amines that was demonstrated by the Klibanov group is based on the use of proteases [4,5]. The protease from subtilisin Carlsberg in combination with trifluoroethyl butyrate as acylating agent turned out to be a suitable enzyme giving enantioselectivities in the range of 60 to 99% ee, whereas several lipases were inefficient. An example for an efficient resolution in the presence of lipases by means of an acylation was reported by Ito and Nemori, applying immobilized enzymes [6]. Immobilization turned out to be a prerequisite for high enantioselectivities. Accordingly, enantioselectivities of 90 to 95% ee were obtained with immobilized enzymes compared with significantly lower 63 to 68% ee when using non-immobilized enzymes.

A wide range of work has been done using “standard” acyl donors such as ethyl acetate or homologous alkyl derivatives thereof, and high enantioselectivities can be obtained in many cases when using, for example, ethyl acetate itself or butyl acetate. The first efficient and general example in this field was reported by the Reetz group. Ethyl acetate functions as an acyl donor. As an enzyme, the lipase from *Candida antarctica* was used and gave high enantioselectivities in the range of 90 to 98% ee for the formed amide, (*R*)-**4** (Scheme 34.3). The conversions were in the range of 20 to 44%. However, high enzyme loadings were used and reaction times varied in a range of 7 to 60 h [7]. Selected examples are given in Scheme 34.3.

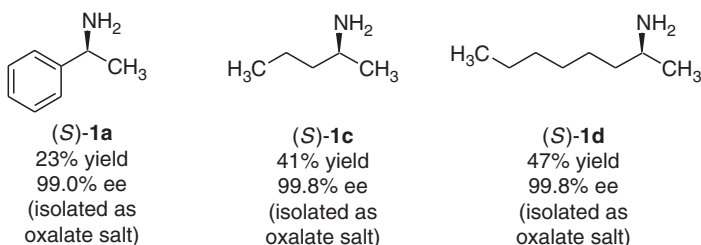
This method of enantioselective acylation of racemic amines using ethyl acetate as a cheap acyl donor was further studied in detail and optimized by the Davis group (Scheme 34.4) [8]. When carrying out the reaction in ethyl acetate as a solvent and in the presence of lipase from *C. antarctica*, a range of amines (*S*)-**1**, comprising aliphatic and aromatic products, were obtained in yields of up to 47%, and with excellent enantioselectivities of up to 99% ee with preference for the (*S*)-enantiomer. A downstream processing for the remaining optically active (*S*)-enantiomers, (*S*)-**1**—by means of precipitation of the corresponding oxalate



SCHEME 34.3



Selected examples

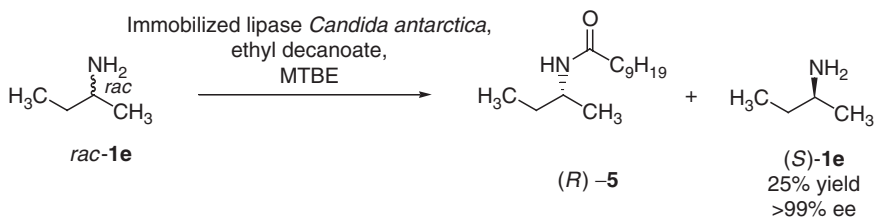


SCHEME 34.4

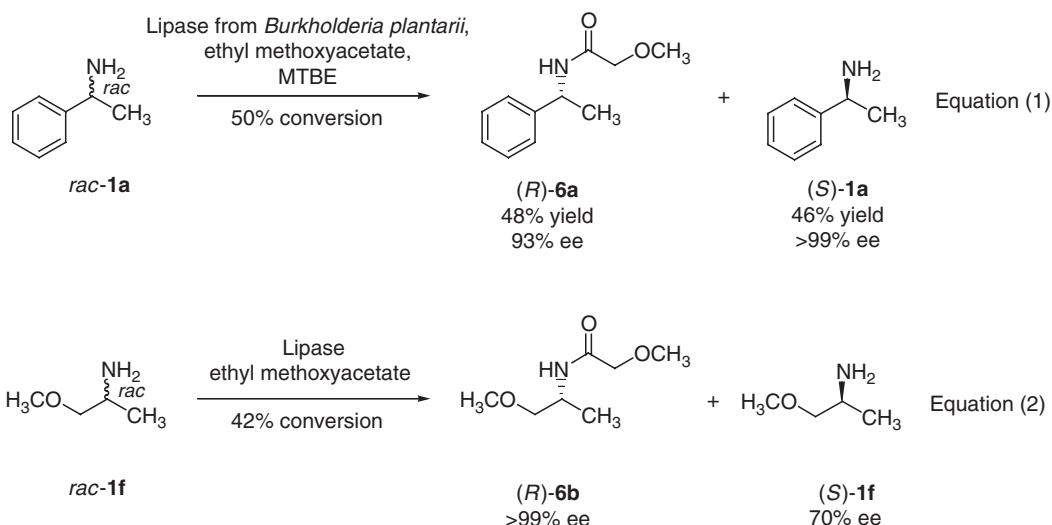
salts—has also been developed. Reaction time, however, was long with 5 to 21 days. Compared with other solvents, reaction in ethyl acetate was faster, although enantioselectivity was somewhat lower in some cases.

The impact of the chain-length of the acyl donor was demonstrated by Patel et al. for the resolution of *rac*-2-butylamine, *rac*-**1e**, using the lipase from *C. antarctica* [9]. Due to low stereodifferentiation between the two similar substituents methyl and ethyl attached to the carbon atom bearing the amino group, low enantioselectivity was obtained when using ethyl butyrate ($E < 10$) as an acyl donor. However, the use of ethyl decanoate as acyl donor and methyl *tert*-butyl ether (MTBE) as a solvent led to an increase of the enantioselectivities of up to 99% at a yield of 25% for (*S*)-**1e** (Scheme 34.5) [9]. In addition, benzyl esters have been used as donors for the resolution of racemic amines [10].

A drawback of all types of alkyl ester-type acyl donors, however, is the low reaction rate in the acylation process, thus being the limiting factor with respect to commercial applications. The breakthrough with respect to industrial applications in the acylation was achieved by BASF researchers, using ethyl methoxyacetate as an acyl donor in the presence of lipases [11]. As a biocatalyst, the lipase from *Burkholderia plantarii*, which shows a high activity of 1000 U/mg for the crude enzyme product, turned out to be particularly useful. Preferred solvents are ethers, in particular MTBE. In the presence of methoxyacetate, the acylation reaction of *rac*-phenylethyl-1-amine, *rac*-**1a**, proceeds very fast, and the initial reaction rates



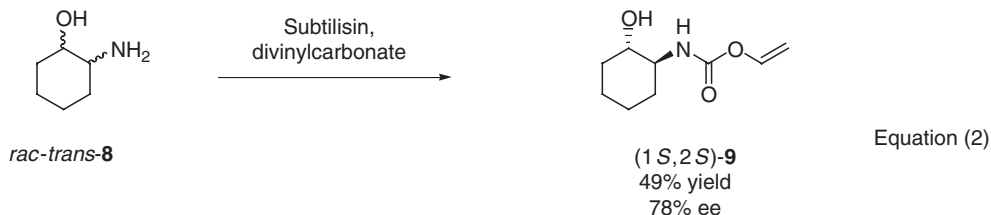
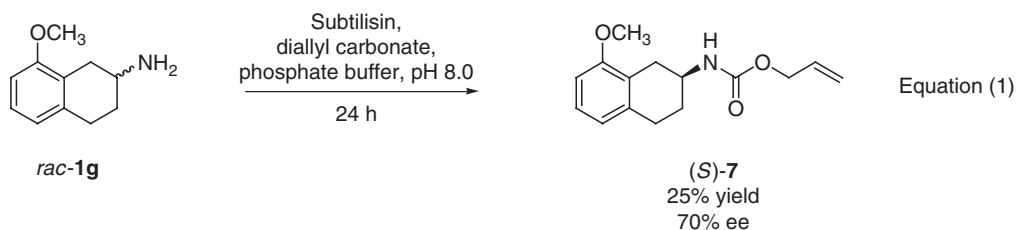
SCHEME 34.5



SCHEME 34.6

are 100 times higher compared with the one when using ethyl butyrate. After reaching a quantitative conversion of 50%, downstream processing gave the remaining (*S*)-amine, (*S*)-**1a**, in 46% yield with a high enantioselectivity of >99% ee (Scheme 34.6, Equation (1)). The corresponding amide, (*R*)-**6a**, is obtained in 48% yield, with 93% ee. The reaction also proceeds highly efficiently with other type of substrates, allowing an efficient access to numerous (*S*)-amines (*S*)-**1**, as well as—after subsequent deacylation—(*R*)-amines. Notably, the enzymatic kinetic resolution of racemic amines is one of the most impressive examples demonstrating how efficient a resolution process can be in spite of the limitation of 50% conversion. The major industrial application of this lipase resolution is the production of (*S*)-methoxyisopropylamine (*S*)-**1f** (Scheme 34.6, Equation (2)), which is an intermediate in the production of the herbicide “Outlook,” with an annual production capacity of 2500 t [12,13]. Furthermore, the resolution of several optically active amines under cGMP conditions is carried out at an annual scale of 1500 t [13]. It should be added that the use of ethyl methoxyacetate as an acyl donor was also reported by Bayer researchers. In their studies, a lipase from *C. antarctica* was used as a biocatalyst [14]. In addition, the Reetz group developed a sol–gel encapsulated form of the lipase from *C. antarctica*, which is suitable to resolve racemic 1-phenylethylamine with 50% conversion (within 29 h) and high enantioselectivity ($E > 100$) [15]. This catalyst is more active than the commercial powder; it showed constant performance when recycled within five reaction cycles. Jaeger et al. also demonstrated cloning and overexpression of a lipase from *Pseudomonas aeruginosa* in a lipase-negative *P. aeruginosa* strain. The strain secreted the recombinant lipase, which turned out to be highly efficient in several resolution processes, including resolution of racemic 2-pentylamine (50% conversion, 96% ee) [16].

In addition to acetates and substituted derivatives thereof, other types of acyl donors have been applied as well. The kinetic resolution of racemic amines using dialkyl or diallyl carbonates has been reported by the Wong group (see, e.g., Scheme 34.7, Equation (1)) [17]. The “nonsymmetrical” substituted phenyl allyl carbonate was found to be a new useful acylation agent for the lipase-catalyzed resolution of 1-methyltetrahydroisochinoline [18]. Alkoxyacylation of racemic amines by a lipase of *C. rugosa* was studied with different lipase samples from *C. rugosa* that are based on cultivation with different inducers and carbon



SCHEME 34.7

sources [19]. Enantioselective acylation was also done with divinyl carbonate as a donor in the presence of subtilisin as a hydrolase; functionalized amines were resolved as well. The subtilisin-catalyzed resolution of *rac-trans*-1-amino-1-hydroxycyclohexane, *rac-trans*-**8**, led to the formation of the corresponding enantiomerically enriched *N*-acylated (1*S*,2*S*)-enantiomer (1*S*,2*S*)-**9** in 49% yield with 78% ee (Scheme 34.7, Equation (2)) [20].

Furthermore, methyl acrylates have been used as acyl donors for the resolution of aliphatic amines [21]. Although enantioselectivities of up to 95% were obtained for the remaining optically active amines, reaction times were rather long with 7 to 11 days, and yields were in the range of 20 to 40%. Another acyl donor applied in the enzymatic acylation of racemic amines and amino alcohols is cyanomethyl pent-4-enoate, which was reported by the Wong group [22].

An elegant approach demonstrating that “nonactivated” acyl donors, namely carboxylic acids, can also be used was reported by Montet et al. (in organic solvents) [23], as well as Kato et al. (under neat conditions and in ionic liquids). Compared with neat reaction conditions, ionic liquids turned out to be superior [24]. Interestingly, the choice of preferred ionic liquid depends on the type of amine. The lipase-catalyzed kinetic resolution in 1-butyl-2,3-dimethylimidazolium trifluoromethanesulfate was also done on preparative scale.

In addition to intensive process development with respect to a high reaction rate, detailed study of the impact of the acyl group on the enzymatic process, and reaction media engineering, a major focus has been on the investigation of the substrate range [25,26]. Among “typical” nonfunctionalized amines, aliphatic as well as aromatic amines are accepted (see also section above). Lipases, however, have been used not only for resolution of nonfunctionalized amines but also for amines bearing other functionalized groups. For example, an enzymatic resolution of a variety of 1-heteroarylamines through enantioselective acylation proceeds very efficiently in the presence of the *C. antarctica* lipase B [27]. High yields and high enantioselectivities in the range of 90 to 99% are obtained. An example for amines that are further functionalized are β -amino esters such as ethyl-3-aminobutyrate. The Gotor group demonstrated that such types of amines could be efficiently resolved by means of a lipase-mediated acylation. In the presence of the lipase from *C. antarctica* and ethyl acetate as both acyl donor and solvent, the remaining amine was obtained in 45% yield with a high

enantioselectivity of 99% ee [28]. The products can be further converted into optically active β -amino acids, which are of pharmaceutical interest.

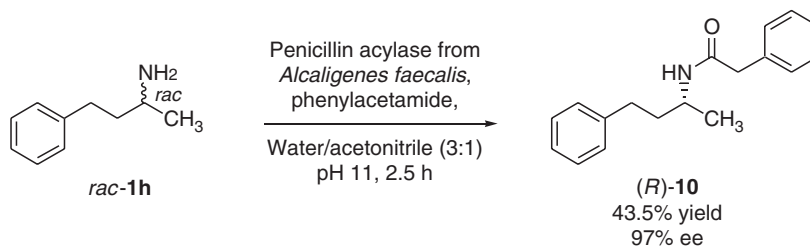
34.2.3 PENICILLIN ACYLASE-CATALYZED RESOLUTION OF RACEMIC AMINES THROUGH ACYLATION

The popularity of the penicillin acylase is not only because of its use as an efficient catalyst for the industrial production of 6-aminopenicillanic acid (6-APA) [29], but also due to its broad utility range with respect to the substitution pattern at the amino functionality. In addition to many amino acids and derivatives that are accepted as substrates in the acylation process (or their corresponding amides in the hydrolytic resolution) [30], several groups demonstrated that penicillin acylase is suitable for the resolution of racemic amines [31,32]. The penicillin acylase from *Alcaligenes faecalis* [33] was successfully applied for the enantioselective acylation of racemic amines using phenylacetamide as an acylating agent. The reaction was carried out at a high pH of 11 in order to ensure deprotonation of the amines [32]. The use of organic cosolvents turned out to be beneficial. Compared with pure aqueous media, a remarkable increase of the enantioselectivity was found when carrying out the resolution of aromatic amines in an aqueous solution with an acetonitrile content of 10 to 25%. Thus, an increase of the *E* value from 110 to 220 was observed for the resolution of 4-phenylbutan-2-amine in an acetonitrile-containing solution (10%), while the reactivity was maintained in the same range. A representative example of the penicillin acylase-based resolution is shown in Scheme 34.8. In contrast, enantioselectivities of analog aliphatic amines were rather low with *E* values below 8. It should be added that the penicillin acylase from *Escherichia coli* gave less satisfactory results.

34.2.4 LIPASE-CATALYZED RESOLUTION OF RACEMIC AMIDES THROUGH HYDROLYSIS

Although most of the lipase-based resolutions for the syntheses of optically active amines are related to the acylation of racemic amines, several examples for the reverse reaction, namely the hydrolysis of racemic *N*-acetylated amines, are also known. There has been a considerable interest from industry on this type of reaction, which is underlined by several contributions from different companies. At the beginning of the 1990s, Shell researchers reported the hydrolysis of *N*-acetylated *rac*-phenylethyl-1-amine in the presence of whole cells from *Arthrobacter* sp., giving access to the enantiomerically pure (*S*)-amine as well as the (*R*)-amide with a high enantioselectivity of >99.5% [34].

Another early example for this type of reaction is the use of an arylalkyl acylamidase for the preparation of both the (*S*)- and (*R*)-forms of the corresponding desired enantiomeric forms of the amines [35]. The (*S*)-enantiomers of phenylethyl-1-amine and 4-phenylbutyl-2-amine were synthesized with high enantioselectivity with several microbes, e.g., *Nocardia*

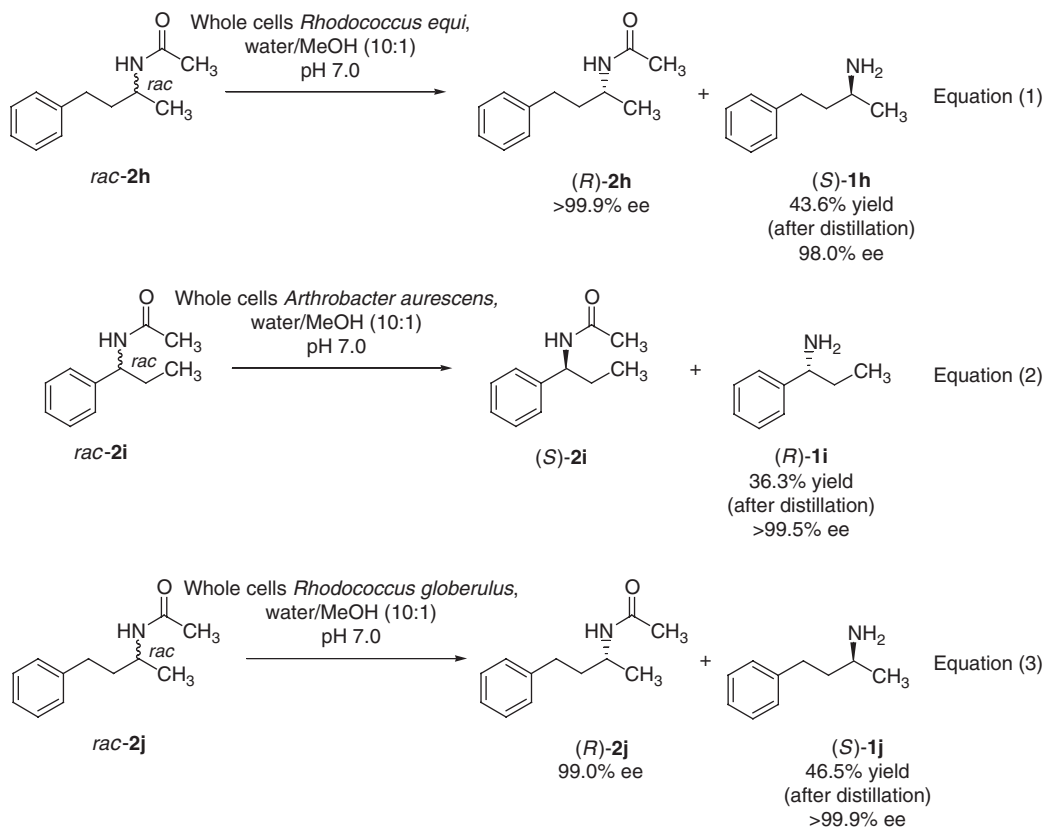


SCHEME 34.8

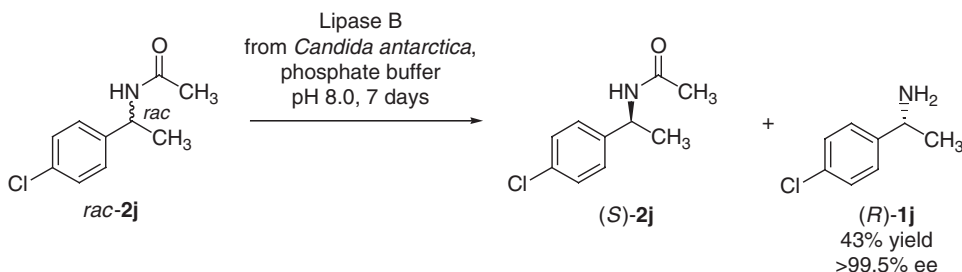
erythropolis. The corresponding (*R*)-enantiomer of 4-phenylbutyl-2-amine was obtained when using a crude amidase from *P. putida*. Microbial hydrolysis starting from the corresponding *N*-acyl derivative has also been reported for the production of D-aminobutanol [36].

Furthermore, three new, stable amidohydrolases and their successful application in the synthesis of several amines as pharmaceutical building blocks have recently been reported by a research group from Novartis [37]. After discovering these (*S*)-selective enzymes from *Rhodococcus equi* and *R. globerulus*, as well as the (*R*)-selective enzyme from *Arthrobacter aurescens* in a screening program, their production was optimized, demonstrating a high operational stability. All of these enzymes showed an excellent enantioselectivity with *E* values of >500 for the desired target reactions. Selected examples are given in Scheme 34.9. The feasibility of the synthesis of (*S*)-2-amino-1-(4-chlorophenyl)-4-pentene, (*S*)-**1j**, has already been successfully demonstrated on a laboratory pilot scale of 20 L.

A highly enantioselective hydrolytic process of *N*-acetylated amines based on the use of lipase B from *C. antarctica* has been developed by Bayer researchers [38]. A representative example is shown in Scheme 34.10. For the resolution of *N*-acetylated *rac*-4-chlorophenylethyl-1-amine, *rac*-**2j**, a very good yield of 43% with an excellent enantioselectivity of >99.5% was obtained for the product (*R*)-4-chlorophenylethyl-1-amine, (*R*)-**1j**. However, the reaction, which had been carried out in pure aqueous buffer media at pH 8 and 50°C, required a long reaction time of 7 days.



SCHEME 34.9



SCHEME 34.10

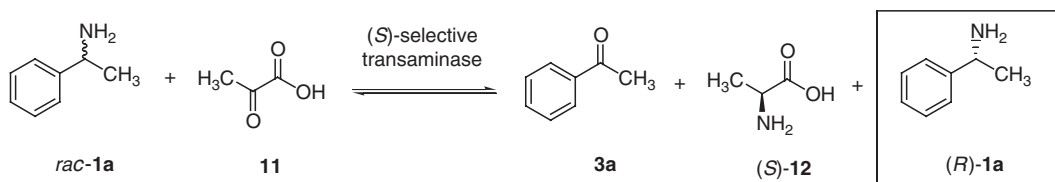
34.2.5 AMINE OXIDASE-CATALYZED RESOLUTION OF RACEMIC AMINES THROUGH OXIDATION

In spite of acylation and hydrolytic processes, selective oxidation of one enantiomer with an amine oxidase can also represent a useful tool for the synthesis of optically active amines. The ability of these enzymes to selectively oxidize amine enantiomers has been known for a long time [39], although preparative applications for the synthesis of optically active amines via this method have only recently been reported. In particular, this process has become an interesting key step in the deracemization of racemic amines, and will be discussed in more detail in the section “Dynamic Kinetic Resolution and Deracemization.”

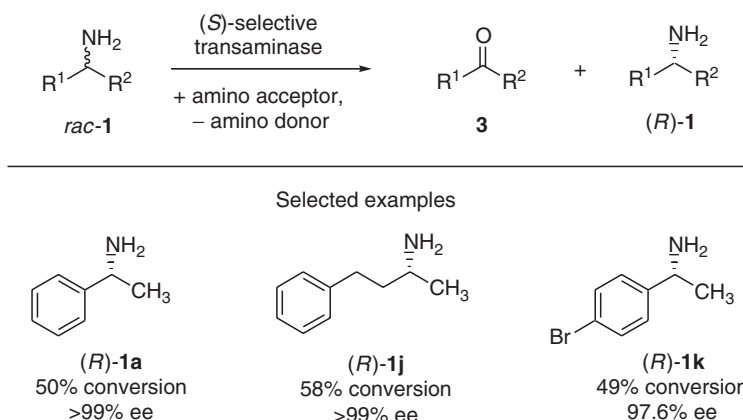
34.2.6 TRANSAMINASE-CATALYZED RESOLUTION OF RACEMIC AMINES THROUGH OXIDATION

An early example of a transaminase-catalyzed resolution of racemic amines [40] is described in a Celgene patent application published in 1990 [41,42]. The optically active amines have been prepared by using a pyridoxal phosphate-dependent ω -amino acid transaminase of *Bacillus* or *Pseudomonas* in the presence of a carbonyl compound such as pyruvate **11** as an amino group acceptor. The (*S*)-amine enantiomer is oxidized to the corresponding ketone **3** (while the pyruvate is converted into L-alanine **12**). Thus, the desired amine enantiomer (*R*)-**1** remained in optically active form. For example, the (*S*)-enantiomer of *rac*-1-phenyl-3-amino butane was transformed 20-fold faster than the corresponding (*R*)-enantiomer. The reaction principle, exemplified for *rac*-phenylethyl-1-amine as a donor and pyruvate as an acceptor, is shown in Scheme 34.11. The choice of the amino acceptor is of importance since pyruvate or oxaloacetate gave fivefold faster reaction rates compared with butan-2-one.

By means of this scalable transaminase technology, a wide range of products are accessible, and pharmaceutically interesting, substituted phenylethyl-1-amines have also been made available in developmental (kg) quantities [42]. Several examples of this efficient resolution technology by means of transaminases are given in Scheme 34.12. Independent of the substitution pattern, excellent enantioselectivities of up to 99% have been obtained for a broad range of aryl-containing amines. For example, resolution of *rac*-phenylethyl-1-amine *rac*-**1a** proceeds with 50% conversion furnishing the optically active (*S*)-phenylethyl-



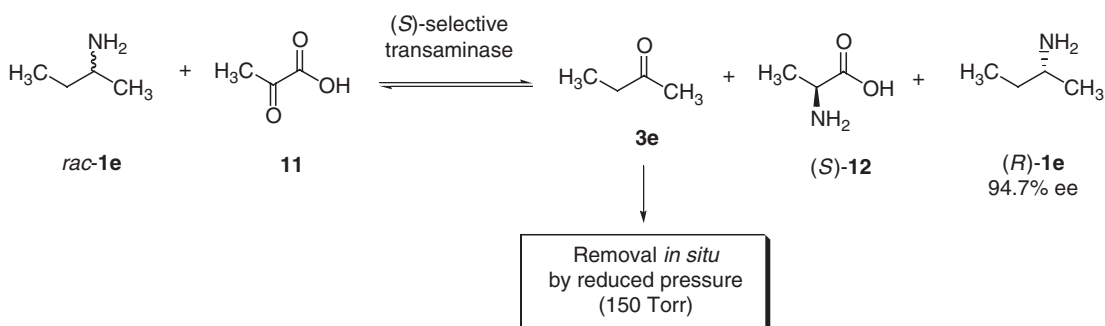
SCHEME 34.11



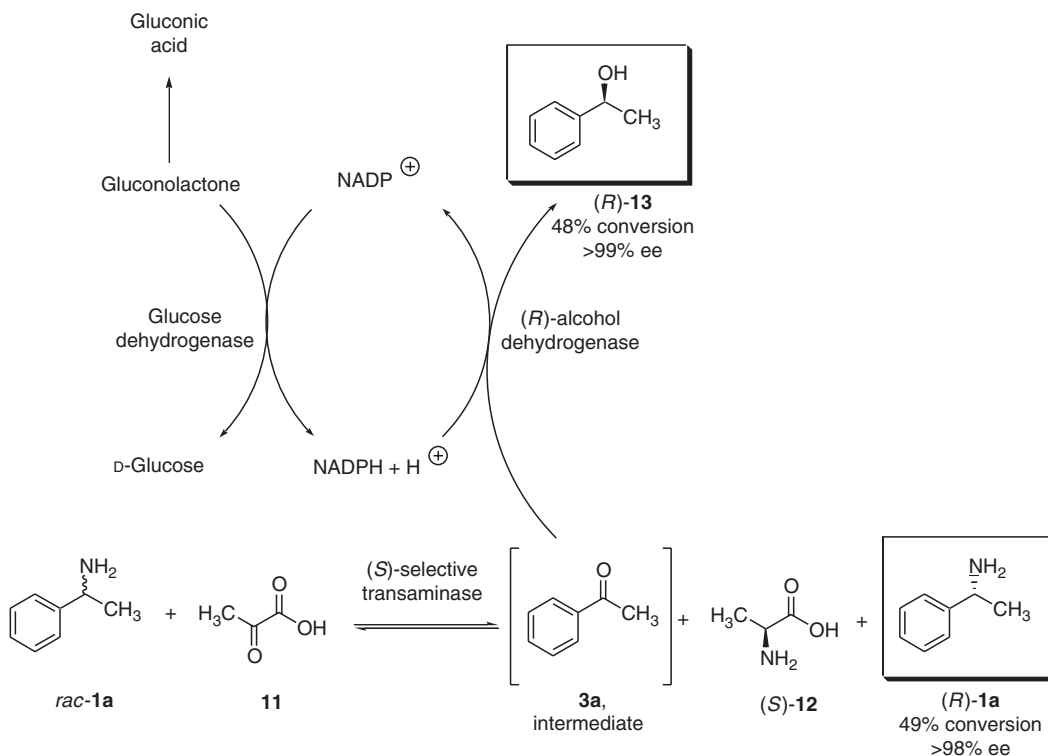
SCHEME 34.12

1-amine, (*S*)-**1a**, with a high enantioselectivity of >99%. In addition to (*S*)-selective transaminases, the analog (*R*)-selective enzymes have been developed as well.

A detailed study on the properties of three ω -transaminases from different microorganisms such as *Klebsiella pneumoniae*, *Bacillus thuringiensis*, and *Vibrio fluvialis* has been done by the Kim group [43]. All three transaminases showed high enantioselectivity when using *rac*-phenylethyl-1-amine as a substrate with *E* values of >50. Substrate tolerance is broad, accepting aryl as well as aliphatic amines. As an amino acceptor, alkyl aldehydes, e.g., propionaldehyde, can be used besides pyruvate. However, a limiting factor in synthetic applications is significant substrate inhibition by (*S*)-phenylethyl-1-amine at concentrations exceeding 200 mM. Acetophenone shows a significant product inhibition too. As transaminase, the one from *V. fluvialis* turned out to be preferred, and has been successfully used in the resolution of aliphatic amines. For example, resolution of *rac*-2-butylamine used at a substrate concentration of 20 mM gave an enantioselectivity of 94.7% after 12 h of reaction time (Scheme 34.13). The reaction was carried out under reduced pressure to selectively remove the by-product 2-butanone, thus suppressing the effect of product inhibitions. Another method to overcome the severe product inhibitions has also been reported by the Kim group for the syntheses of optically active aryl amines by means of a biphasic system [44]. Application of an enzyme-membrane reactor has also proven to be an efficient tool for the kinetic resolution of amines with a transaminase [45].



SCHEME 34.13

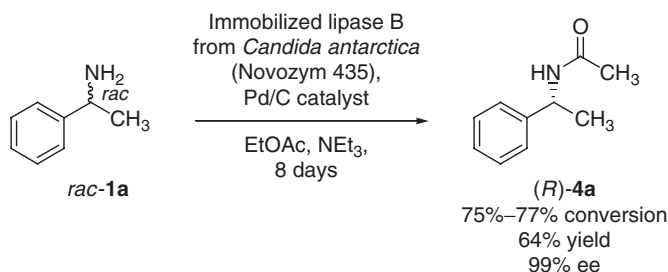


SCHEME 34.14

A unique resolution concept has recently been developed by the Kim group, coupling a resolution of a racemic amine with an enantioselective synthesis of an optically active alcohol [46]. One enantiomer of the amine is oxidized to the ketone, and subsequently reduced by means of an alcohol dehydrogenase-catalyzed reduction process under formation of the corresponding optically active alcohol. By means of this simultaneous reduction process, product inhibition of the formed ketone can be suppressed allowing an efficient synthesis of both the desired (*R*)-amine and (*R*)-phenylethan-1-ol. The cofactor regeneration of the ketone reduction process was carried out through a glucose dehydrogenase-catalyzed oxidation of D-glucose under recycling of the required reduced form of the cofactor, NADPH. The concept—exemplified by the resolution of *rac*-phenylethyl-1-amine—is shown in Scheme 34.14. At a substrate concentration of 100 mM of *rac*-phenylethyl-1-amine, a high conversion was observed, leading to the corresponding (*R*)-phenylethyl-1-amine in 49% and (*R*)-phenylethan-1-ol in 48% within a reaction time of 18 h.

34.3 DYNAMIC KINETIC RESOLUTION AND DERACEMIZATION

A prerequisite for dynamic kinetic resolutions is a sufficient *in situ* racemization process in combination with the applied resolution process. This has been achieved for lipase-based resolution processes by several groups. Deracemization processes also allow the efficient transformation of a racemic compound into an enantiomerically pure form with (theoretically) quantitative conversion. Both types of concepts, dynamic kinetic resolutions (Route (D) in Scheme 34.1) and deracemization (Route (E) in Scheme 34.1), are described in more detail in the following section.



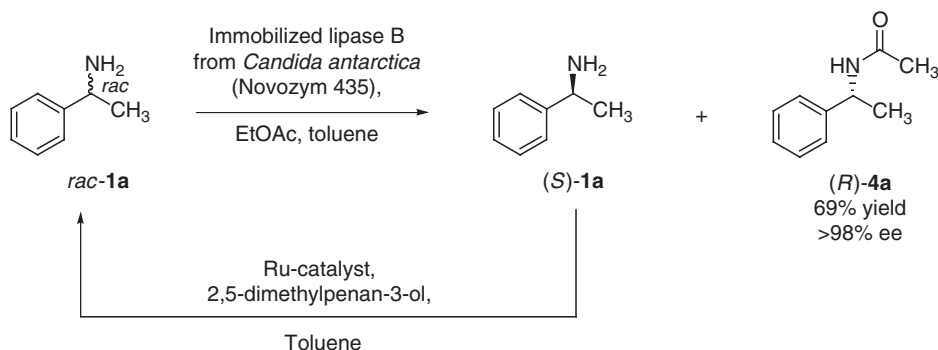
SCHEME 34.15

34.3.1 LIPASE-CATALYZED DYNAMIC KINETIC RESOLUTION OF RACEMIC AMINES

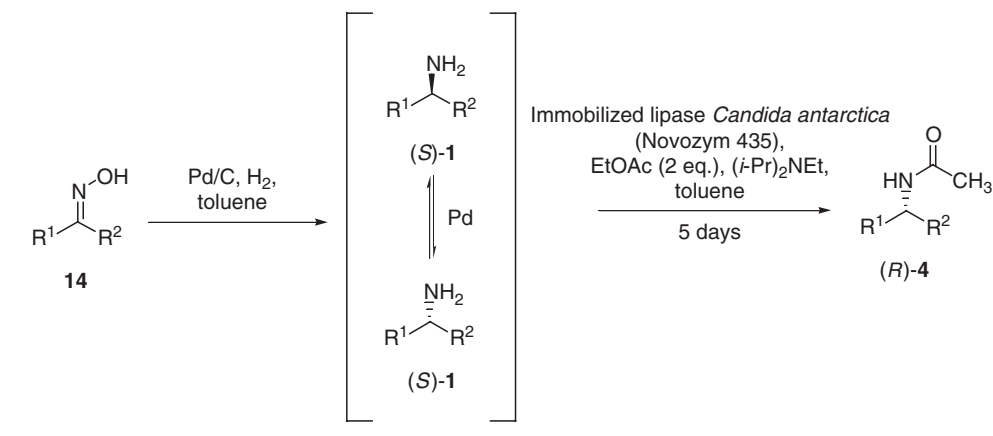
The extension of the lipase resolution toward a dynamic kinetic resolution process has been developed by several groups. The first example was reported by the Reetz group, applying Pd/C as a metal catalyst for the racemization of the amine in combination with the enzymatic acylation of a racemic amine [47]. As an enzyme, the lipase Novozym 435 (*C. antarctica* lipase B) was used. After a reaction time of 8 days, the desired optically active amides were formed with conversions of 75 to 77%. The enantioselectivity was excellent, with 99% ee for the synthesis of *N*-acetylated (*R*)-phenylethyl-1-amine (Scheme 34.15).

The Bäckvall group studied mild racemization conditions for optically active amines, and developed a highly efficient metal catalytic procedure based on a ruthenium complex for this purpose [48,49]. The racemization protocol is very general with respect to substrate range, and also led to high recovery yields of the amine in the range of 95 to 99%. This racemization process was combined with a lipase-based esterification in a two-step manner for the synthesis of (*R*)-*N*-(1-phenylethyl)acetamide, (*R*)-4a. As an enzyme catalyst, the lipase B from *C. antarctica* was applied, and ethyl acetate used as a donor. After two resolutions and one racemization, the desired product was obtained in a yield of 69% with a high ee of >98% (Scheme 34.16).

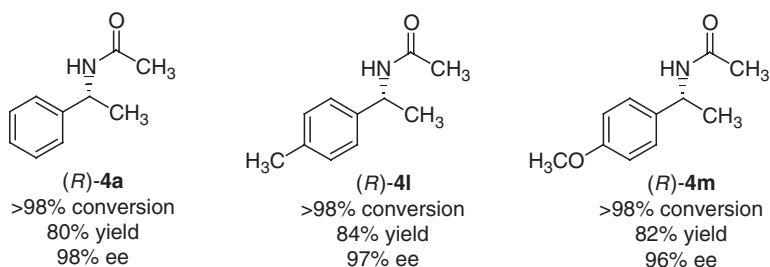
Another dynamic kinetic resolution that is based on the use of ketoximes as precursor has been developed by the Kim group [50,51]. The one-pot synthesis concept, which is shown in Scheme 34.17, is based on the use of two catalysts, namely palladium and a lipase. Whereas the biocatalyst—an immobilized *C. antarctica* B lipase—selectively catalyzes the acylation of one amine enantiomer, the palladium catalyst is required for the initial conversion of the ketoxime into the racemic amine, as well as for the subsequent racemization of the amine. Typically the reactions were carried out in toluene at a reaction temperature of 60°C, with a



SCHEME 34.16



Selected examples



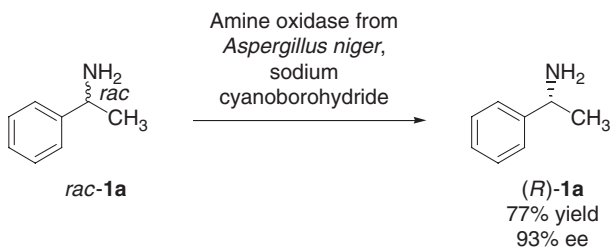
SCHEME 34.17

reaction time of 5 days. Several examples are shown in Scheme 34.17. In addition to acyclic ketoximes, cyclic analogs thereof can be used. In general, excellent conversions of >98% accompanied by high enantioselectivities in the range of 94 to 99% ee were obtained. After purification, the desired (R)-amine products were obtained in yields of 70 to 98%.

Thus, highly enantioselective dynamic kinetic resolution methodologies of racemic amines are available. However, a challenge for the future certainly remains the improvement of the reaction time, which is still in the range of several days.

34.3.2 AMINE OXIDASE-CATALYZED DERACEMIZATION OF RACEMIC AMINES

Deracemization reactions play an important role in the synthesis of optically active compounds, comprising those bearing a secondary alcohol as well as amino functionality [52]. Whereas several examples of applications of amine oxidases for deracemization processes in α -amino acid synthesis have already been reported [53], their efficient extension toward the synthesis of amines remained a challenge for a long time. The extension of the amine oxidases processes (see section above) toward a deracemization process has been impressively demonstrated by the Turner group [54]. The deracemization reaction is based on the combination of an enzymatic, highly enantioselective amine oxidation process with a nonasymmetric chemically reductive amination reaction. Both reactions are compatible with each other, thus allowing a one-pot reaction. As a reducing agent, sodium cyanoborohydride was used, which turned out to be more suitable than sodium borohydride. For the synthetic dynamic kinetic resolution process, an optimized mutant of the amine oxidase from *Aspergillus niger* was used. This mutant has been found by *in vitro* evolution technology, and turned out to



SCHEME 34.18

give improved results with respect to both catalytic activity and enantioselectivity in the deracemization of *rac*-1-phenylethylamine. By means of this improved enzyme, the corresponding (*R*)-1-phenylethylamine was formed in 77% yield with an enantioselectivity of 93% ee (Scheme 34.18).

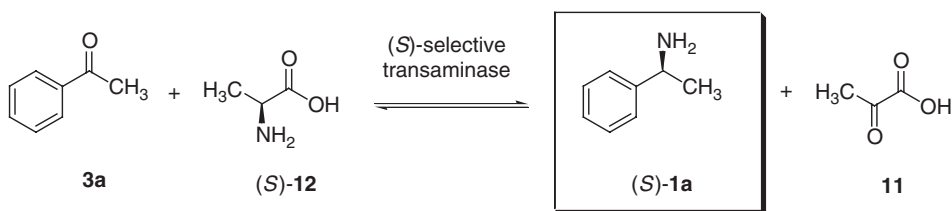
34.4 ASYMMETRIC SYNTHESIS

34.4.1 ASYMMETRIC TRANSAMINATION

The direct conversion of a ketone into the desired chiral amine—shown as Route (F) in Scheme 34.1—probably represents the most straightforward approach toward this class of compounds, as it represents an asymmetric catalytic reaction with (theoretically) 100% conversion. Such a process can be carried out by means of a transamination (originally known for the synthesis of enantiomerically pure α -amino acids starting from keto acids). Several transaminases have been identified that tolerate not only α -keto acids (as a precursor for amino acids) but also ketones, thus offering the potential to synthesize the corresponding optically active amines. In addition to the transaminase resolution of racemic amines with keto acids as an “amino acceptor,” the first efficient syntheses of direct asymmetric transaminations have been reported in the early 1990s by Celgene researchers. The basic concept of the transamination process, which is shown in Scheme 34.19—exemplified for the synthesis of (*S*)-phenylethyl-1-amine, (*S*)-**1a**, using L-alanine as an amine donor—is based on the transformation of the ketone into the optically active amine under simultaneous conversion of an amino donor (L-amino acid in this case) into its carbonyl derivative (keto acid in this case) [41,42]. Alternatively, isopropylamine can also be used as an amine donor instead of an L-amino acid [55].

The key step, however, is not only the transamination process but, particularly, the issue of how to shift the equilibrium to the (thermodynamically nonpreferred) direction of amino and keto acids. Accordingly, the resolution of racemic amines using a keto acid as an “amino acceptor” is more favored and proceeds with high conversion without needing further process steps. In contrast, removing a formed product—preferably the formed carbonyl by-product—from the reaction mixture is essential for an asymmetric transamination process according to Scheme 34.20. Several options are conceivable, most of them addressing a way to decompose the oxidation product formed by deamination of the amine donor. Alternatively, removal of the formed keto product by evaporation has also been efficiently done, especially when isopropylamine has been used as an amine donor under formation of acetone.

A highly efficient application of the transamination concept based on isopropylamine as a donor was reported by the Matcham group for the synthesis of (*S*)-methoxyisopropylamine [56], which is of commercial relevance as it can be used as an intermediate for the production

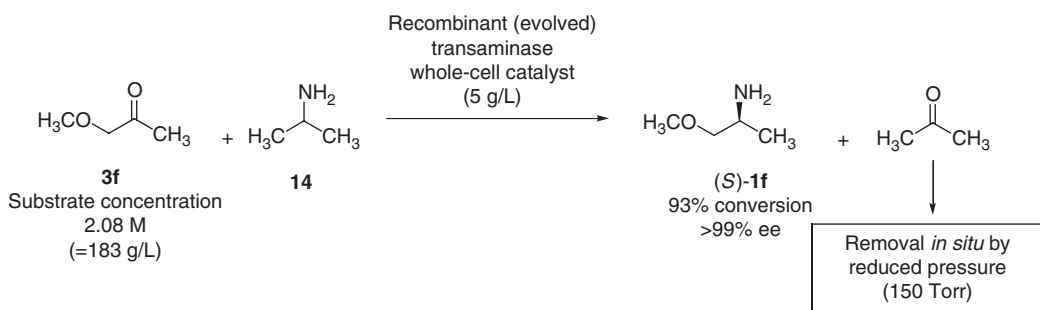


SCHEME 34.19

of herbicides such as (*S*)-dimethenamid and (*S*)-metolachlor. The latter is produced in quantities of 10,000 t/plant through a metal-catalyzed hydrogenation process, which, however, provides the product in only 79% ee. Choosing the transaminase route, Matcham et al. succeeded—after optimization of the enzyme catalyst through directed evolution as well as process conditions—in the formation of the desired product (*S*)-methoxyisopropylamine with 93% conversion when removing the acetone during the reaction. The reactions were run under vacuum (100 mm Hg) at 50°C, and loss of methoxyacetone was avoided by means of an overhead condenser. The substrate concentration was at an impressive 2.08 M, corresponding to a substrate input of 183 g/L. The enantioselectivity of this process is excellent with >99% ee (Scheme 34.20). As a biocatalyst, recombinant whole cells bearing an optimized transaminase mutant with respect to thermal and chemical stabilities were used at a catalytic amount of 5 g/L.

Celgene also applied its transamination technology for numerous other types of optically active amines, comprising both (*R*)- and (*S*)-enantiomers. This transamination technology has already been scaled up to the production of optically active amines on a >500 kg scale [55].

The use of L-alanine as an alternative α-amino acid donor has also been investigated by the Kim group [57]. In order to shift the reaction toward the product side, removal of the formed keto acid as a side product turned out to be essential. When using crude extracts with a transaminase, removal of pyruvate was achieved by means of additionally added lactate dehydrogenase (LDH). The preferred way that was found, however, consisted of a whole-cell approach under consumption of the formed pyruvate within the cell metabolism as well as secretion of the synthesized (*S*)-amine. In the presence of whole cells, (*S*)-phenylethyl-1-amine was formed with a conversion of 90% and an enantioselectivity of >99% ee after a reaction time of 1 day. The substrate concentration of acetophenone, however, was 30 mM, and thus requires further optimization with respect to a technical application.



SCHEME 34.20

34.5 SUMMARY

For the development of synthetic methods for optically active amines, which are key building blocks for a variety of drugs and agrochemicals, numerous biocatalytic methods are available. Interestingly, these methods are based on various synthetic concepts such as (i) several types of kinetic resolutions using hydrolases, amine oxidases, and transaminases; (ii) dynamic kinetic resolutions with hydrolases; (iii) deracemization with amine oxidases; and (iv) asymmetric catalytic synthesis using transaminases. In addition to the broad variety of synthetic tools, it is further impressive that some technologies already made the “jump” from a laboratory scale application to a technically applied production process. In particular, the lipase-based acylation of amines, which is carried out at BASF on industrial scale, represents a highly efficient method for the large-scale manufacture of amines. Furthermore, this technology underlines that kinetic resolutions—although restricted in principle by a maximum yield of 50%—can be highly competitive on industrial scale, e.g., when achieving high volumetric productivities in combination with an efficient recycling of the undesired enantiomer. Another impressive technology already proven on a large scale is the transaminase technology, which has been developed at Celgene.

Thus, the biocatalytic syntheses of optically active amines are already valuable tools in the pharmaceutical and biotechnological industries for the synthesis of the desired amine target molecules. It can be expected that their importance will further increase in the future. Among the major challenges for the future might be the improvement of the volumetric productivity of the dynamic kinetic resolutions by combining enzymes (for resolution) and metal catalysts (for racemization).

REFERENCES

1. For a review, see Gotor, V., *Bioorg. Med. Chem.*, 7, 2189, 1999.
2. In addition to biocatalytic approaches, several highly efficient chemocatalytic syntheses have been developed. Selected recent excellent examples are: (a) asymmetric resolution of racemic amines: Arai, S., Bellemin-Lapponnaz, S., and Fu, G., *Angew. Chem.*, 113, 242, 2001; (b) asymmetric hydrogenation of *N*-phosphinoyl imines: Spindler, F. and Blaser, H.-U., *Adv. Synth. Catal.*, 343, 68, 2001; (c) asymmetric hydrogenation of imines: Cobley, C.J. and Henschke, J.P., *Adv. Synth. Catal.*, 345, 195, 2001.
3. Reviews for enantioselective biocatalytic amino acid syntheses: (a) α -Amino acids: Gröger, H. and Drauz, K., in *Asymmetric Catalysis on Industrial Scale*, Blaser, H.-U. and Schmidt, E. Eds., Wiley-VCH, Weinheim, Germany, 2003, p. 131; (b) β -Amino acids: Liu, M. and Sibi, M.P., *Tetrahedron*, 58, 7991, 2002.
4. Kitaguchi, H., Fitzpatrick, P.A., Huber, J.E., and Klibanov, A.M., *J. Am. Chem. Soc.*, 111, 3094, 1989.
5. Notably, for some specific amino alcohols the porcine pancreatic lipase was found to be suitable already in 1988 by the Gotor group; see Gotor, V., Brieva, R., and Rebollo, F., *J. Chem. Soc. Chem. Commun.*, 957, 1988.
6. Ito, I. and Nemori, R. (Fuji Photo), JP Pat. Appl. 03191797, 1991.
7. Reetz, M.T. and Dreisbach, C., *Chimia*, 48, 570, 1994.
8. Davis, B.A. and Durden, D.A., *Synth Commun.*, 31, 569, 2001.
9. Goswami, A., Guo, Z., Parker, W.L., and Patel, R.N., *Tetrahedron: Asymmetry*, 16, 1715, 2005.
10. Adamczyk, M. and Grote, J., *Tetrahedron Lett.*, 37, 7913, 1996.
11. Balkenhohl, F., Ditrich, K., Hauer, B., and Ladner, W., *J. Prakt. Chem.*, 339, 381, 1997.
12. Review: Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M., and Witholt, B., *Nature*, 409, 258, 2001.
13. Review: Breuer, M., Ditrich, K., Habicher, T., Hauer, B., Keßler, M., Stürmer, R., and Zelinski, T., *Angew. Chem.*, 116, 806, 2004.
14. Steltzer, U. and Dreisbach, C., DE Pat. 19637336, 1996.

15. Reetz, M.T., Tielmann, P., Wiesenhöfer, W., Könen, W., and Zonta, A., *Adv. Synth. Catal.*, 345, 717, 2003.
16. Jaeger, K.-E., Liebeton, K., Zonta, A., Schimmosek, K., and Reetz, M.T., *Appl. Microbiol. Biotechnol.*, 46, 99, 1996.
17. Wong, C.-H., Orsat, B., Moree, W.J., and Takayama, S., U.S. Pat. Appl. 5981267, 1999.
18. Breen, G.F., *Tetrahedron: Asymmetry*, 15, 1427, 2004.
19. Alcantara, A.R., Dominguez de Maria, P., Fernandez, M., Hernaiz, M.J., Sanchez-Montero, J.M., and Sinisterra, J.V., *Food Technol. Biotechnol.*, 42, 343, 2004.
20. Orsat, B., Alper, P.B., Moree, W., Mak, C.-P., and Wong, C.-H., *J. Am. Chem. Soc.*, 118, 712, 1996.
21. Puertas, S., Brieva, R., Rebolledo, F., and Gotor, V., *Tetrahedron*, 49, 4007, 1993.
22. Takayama, S., Moree, W.J., and Wong, C.-H., *Tetrahedron Lett.*, 37, 6287, 1996.
23. Montet, D., Pina, M., Graille, J., Renard, G., and Grimaud, J., *Fat. Sci. Technol.*, 91, 14, 1989.
24. (a) Irimescu, R. and Kato, K., *Tetrahedron Lett.*, 45, 523, 2004; (b) Irimescu, R. and Kato, K., *J. Mol. Catal. B.*, 30, 189, 2004.
25. van Rantwijk, F., Hacking, M.A.P.J., and Sheldon, R.A., *Monatsh. Chem.*, 131, 549, 2000.
26. (a) Garcia, M.J., Rebolledo, F., and Gotor, V., *Tetrahedron: Asymmetry*, 3, 1519, 1992; (b) Garcia, M.J., Rebolledo, F., and Gotor, V., *Tetrahedron: Asymmetry*, 4, 2199, 1993; (c) Gotor, V., Menendez, E., Mouloungui, Z., and Gaset, A., *J. Chem. Soc. Perkin Trans.*, 1, 2453, 1993; (d) Quiros, M., Sanchez, V.M., Brieva, R., Rebolledo, F., and Gotor, V., *Tetrahedron: Asymmetry*, 4, 1105, 1993.
27. Skupinska, K.A., McEachern, E.J., Baird, I.R., Skerlj, R.T., and Bridger, G.J., *J. Org. Chem.*, 68, 3546, 2003.
28. Sanchez, V.M., Rebolledo, F., and Gotor, V., *Tetrahedron: Asymmetry*, 8, 37, 1997.
29. Verweij, J. and De Vroom, E., *Recl. Trav. Chim. Pays-Bas*, 112, 66, 1993.
30. α -Amino acids: (a) Lucente, G., Romeo, A., and Rossi, A., *Experiencia*, 21, 317, 1965; (b) Cole, M., *Biochem. J.*, 115, 741, 1969; (c) Kasche, V., Michaelis, G., and Wiesemann, T., *Biochem. Biomed. Acta*, 50, 38, 1991.
31. Rossi, D., Calcagni, A., and Romeo, A., *J. Org. Chem.*, 44, 2222, 1979.
32. van Langen, L.M., Oosthoek, N.H.P., Guranda, D.T., van Rantwijk, F., Svedas, V.K., and Sheldon, R.A., *Tetrahedron: Asymmetry*, 11, 4593, 2000.
33. Svedas, V., Guranda, D., van Langen, L., van Rantwijk, F., Sheldon, R.A., *FEBS Lett.*, 1997, 417, 414, and references mentioned therein.
34. Phillips, J.T. and Shears, J.H. (Shell International Research Maatschappij), EP Pat. 399589, 1989.
35. (a) Shimizu, S., Ogawa, J., and Yamada, H., *Appl. Microbiol. Biotechnol.*, 37, 164, 1992; (b) Ogawa, J., Shimizu, S., and Yamada, H., *Bioorg. Med. Chem.*, 2, 429, 1994; (c) Shimizu, S., Ogawa, J., Chung, M.C.M., and Yamada, H., *Eur. J. Biochem.*, 209, 375, 1992.
36. Chisso Corp., JP Pat. 58198296; *Chem. Abstr.*, 100, 119354, 1997.
37. (a) Laumen, K., Kittelmann, M., and Gishalba, O., *J. Mol. Catal. B: Enzym.*, 19, 55, 2002; (b) Laumen, K., Brunella, A., Graf, M., Kittelmann, M., Walser, P., and Gishalba, O., *Pharmacochemistry Library: Trends in Drug Research II*, Vol. 29, Elsevier, Amsterdam, p. 17, 1998.
38. Smidt, H., Fischer, A., Fischer, P., Schmidt, R.D., and Stelzer, U. (Bayer AG), EP Pat. 812363, 1995.
39. For example, see: Summers, M.C., Markovic, R., and Klinman, J.P., *Biochemistry*, 18, 1969, 1979.
40. Review: Stirling, D.I., in *Chirality in Industry*, Collins, A.N., Sheldrake, G.N., and Crosby, J., Eds., Wiley, New York, 1992.
41. Stirling, D.I., Zeitlin, A.L., and Matcham, G.W. (Celgene Corp.), U.S. Pat. Appl. 4950606, 1990.
42. Matcham, G., *Specialty Chemicals*, 12, 178, 1992.
43. Shin, J.-S. and Kim, B.-G., *Biosci. Biotechnol. Biochem.*, 65, 1782, 2001.
44. Shin, J.-S. and Kim, B.-G., *Biotechnol. Bioeng.*, 55, 348, 1997.
45. Shin, J.-S., Kim, B.-G., Liese, A., and Wandrey, C., *Biotechnol. Bioeng.*, 73, 179, 2001.
46. Yun, H., Yang, Y.-H., Cho, B.-K., Hwang, B.-Y., and Kim, B.-G., *Biotechnol. Lett.*, 25, 809, 2003.
47. Reetz, M.T. and Schimossek, K., *Chimia*, 50, 668, 1996.
48. Pamies, O., Ell, A.H., Samec, J.S.M., Hermanns, N., and Bäckvall, J.-E., *Tetrahedron Lett.*, 43, 4699, 2002.

49. Review about combination of enzymes and metal catalysts in asymmetric catalysis: Pamies, O. and Bäckvall, J.-E., *Chem. Rev.*, 103, 3247, 2003.
50. Choi, Y.K., Kim, M.J., Ahn, Y., and Kim, M.-J., *Org. Lett.*, 3, 4099, 2001.
51. Review: Kim, M.-J., Ahn, Y., and Park, J., *Curr. Opin. Biotechnol.*, 13, 578, 2002.
52. Kroutil, W. and Faber, K., *Tetrahedron: Asymmetry*, 9, 2901, 1998.
53. (a) Hafner, E.W. and Wellner, D., *Proc. Natl. Acad. Sci.*, 68, 987, 1971; (b) Huh, J.W., Yokoigawa, K., Esaki, N., and Soda, K., *J. Ferment. Bioeng.*, 74, 189, 1992; (c) Huh, J.W., Yokoigawa, K., Esaki, N., and Soda, K., *Biosci. Biotech. Biochem.*, 56, 2081, 1992; (d) Soda, K., Oikawa, T., and Yokoigawa, K., *J. Mol. Catal. B: Enzym.*, 11, 149, 2001; (e) Beard, T.M. and Turner, N.J., *Chem. Commun.*, 246, 2002.
54. Alexeeva, M., Enright, A., Dawson, M.J., Mahmoudian, M., and Turner, N.J., *Angew. Chem. Int. Ed.*, 41, 3177, 2002.
55. Matcham, G.G. and Bowen, A.R.S., *Chim. Oggi*, 6, 20, 1996.
56. Matcham, G., Bhatia, M., Lang, W., Lewis, C., Nelson, R., Wang, A., and Wu, W., *Chimia*, 53, 548, 1999.
57. Shin, J.-S. and Kim, B.-G., *Biotechnol. Bioeng.*, 65, 206, 1999.

